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(21) International Application Number: PCT/GB96/01846 (22) International Filing Date: 29 July 1996 (29.07.96) (30) Priority Data: 9516241.8 8 August 1995 (08.08.95) GB (71) Applicant (for all designated States except US): ZENECA LIMITED [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): JEPSON, Ian [GB/GB]; 31 Gringer Hill, Maidenhead, Berkshire SL6 7LY (GB). PAINE, Jacqueline, Ann, Mary [GB/GB]; 5 Frensham, Crown Wood, Bracknell, Berkshire RG12 0TQ (GB). (74) Agents: ROBERTS, Alison, Christine et al.; Zeneca Agrochemicals, Intellectual Property Dept., Jealott's Hill Research Station, P.O. Box 3538, Bracknell, Berkshire RG42 6YA (GB).		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: DNA CONSTRUCTS (57) Abstract A chemically-inducible plant gene expression cassette comprising a first promoter operatively linked to a regulator sequence which is derived from the <i>alcR</i> gene and encodes a regulator protein, and an inducible promoter operatively linked to a target gene which encodes a protein which is damaging to insects or whose expression induces a metabolic pathway which produces a metabolite which is damaging to insects, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene.		

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DNA CONSTRUCTS

The present invention relates to DNA constructs and plants incorporating them. In particular, it relates to promoter sequences and their use in the expression of genes which confer insecticidal activity on plants.

Advances in plant biotechnology have resulted in the generation of transgenic plants which are protected against feeding insect larvae.

Many organisms produce proteins which are harmful to insects and among these is the organism *Bacillus thuringiensis* which produces a crystal-associated protein δ endotoxin which kills insect larvae upon ingestion. It is not, however, toxic to mammals. It is thus very useful as an agricultural insecticide. Many strains of *B. thuringiensis* are active against insect pests, and the genes encoding for the insect endotoxins have been characterised. The *B. thuringiensis* δ endotoxins include those specifically insecticidal to Lepidopteran larvae (such as the CryI type proteins), those specifically insecticidal to Coleopteran larvae (such as the CryIII type proteins) and those with dual specificity for Lepidoptera and Coleoptera (such as CryV). Chimeric proteins comprising at least part of a *B. thuringiensis* endotoxin have also been proposed with the aim of improving the properties of the endotoxin in some way, for example improved speed of kill. Transgenic plants expressing genes which encode for the insecticidal endotoxins are also known.

Other ways of damaging insects include stimulating plant metabolic pathways which produce metabolites which are insecticidal.

We propose a system where genes encoding active insecticidal proteins such as *B. thuringiensis* endotoxins would be expressed in an inducible manner dependent upon application of a specific activating chemical. Alternatively, the induction of pathways which produce metabolites damaging to insects could be achieved. This approach has a number of benefits, including the following:

1. Constitutive expression in plants of insect resistance genes such as *B. thuringiensis* endotoxins, will lead to a significant increase in the selection pressure for resistant insect species. The inducible regulation of insect resistance genes will reduce the risk of development of resistant pests. For example, insecticidal gene expression can be induced only at the point in the growing season where protection is required. In addition,

switchable insect tolerance can be used as a part of an integrated pest management system, in which chemical treatments to induce insecticidal gene expression can be alternated with standard insecticidal pesticide treatments.

2. There is a risk that overexpression, from strong constitutive promoters, could lead to detrimental effects on plant development resulting in aberrant germination flowering or yield penalties. Inducible expression would reduce the risk of detrimental effects as the transgene could be expressed for a short period avoiding sensitive points in development.
3. The switch chemical could be added to standard insecticide formulations to give both a chemical and gene effect, thus killing insects by two independent mechanisms.

We have developed an inducible gene regulation system (gene switch) based on the *alcR* regulatory protein from *Aspergillus nidulans* which activates genes expression from the *alcA* promoter in the presence of certain alcohols and ketones. This system is described in our International Patent Publication No. WO93/21334 which is incorporated herein by reference.

The *alcA/alcR* gene activation system from the fungus *Aspergillus nidulans* is also well characterised. The ethanol utilisation pathway in *A. nidulans* is responsible for the degradation of alcohols and aldehydes. Three genes have been shown to be involved in the ethanol utilisation pathway. Genes *alcA* and *alcR* have been shown to lie close together on linkage group VII and *aldA* maps to linkage group VIII (Pateman JH *et al*, 1984, *Proc. Soc. Lond.*, B217:243-264; Sealy-Lewis HM and Lockington RA, 1984, *Curr. Genet.* 8:253-259). Gene *alcA* encodes ADHI in *A. nidulans* and *aldA* encodes AldDH, the second enzyme responsible for ethanol utilisation. The expression of both *alcA* and *aldA* are induced by ethanol and a number of other inducers (Creaser EH *et al*, 1984, *Biochemical J.*, 255:449-454) via the transcription activator *alcR*. The *alcR* gene and a co-inducer are responsible for the expression of *alcA* and *aldA* since a number of mutations and deletions in *alcR* result in the pleiotropic loss of ADHI and aldDH (Felenbok B *et al*, 1988, *Gene*, 73:385-396; Pateman *et al*, 1984; Sealy-Lewis & Lockington, 1984). The ALCR protein activates expression from *alcA* by binding to three specific sites in the *alcA* promoter (Kulmberg P *et al*, 1992, *J. Biol. Chem.*, 267:21146-21153).

The *alcR* gene was cloned (Lockington RA *et al*, 1985, *Gene*, 33:137-149) and sequenced (Felenbok *et al*, 1988). The expression of the *alcR* gene is inducible, autoregulated and subject to glucose repression mediated by the CREA repressor (Bailey C and Arst HN,

1975, *Eur. J. Biochem.* 51:573-577; Lockington RA *et al*, 1987, *Mol. Microbiology*, 1:275-281; Dowzer CEA and Kelly JM, 1989, *Curr. Genet.* 15:457-459; Dowzer CEA and Kelly JM, 1991, *Mol. Cell. Biol.* 11:5701-5709). The ALCR regulatory protein contains 6 cysteines near its N terminus co-ordinated in a zinc binuclear cluster (Kulmberg P *et al*, 1991, *FEBS Letts.*, 280:11-16). This cluster is related to highly conserved DNA binding domains found in transcription factors of other ascomycetes. Transcription factors GAL4 and LAC9 have been shown to have binuclear complexes which have a cloverleaf type structure containing two Zn(II) atoms (Pan T and Coleman JE, 1990, *Biochemistry*, 29:3023-3029; Halvorsen YDC *et al*, 1990, *J. Biol. Chem.*, 265:13283-13289). The structure of ALCR is similar to this type except for the presence of an asymmetrical loop of 16 residues between Cys-3 and Cys-4. ALCR positively activates expression of itself by binding to two specific sites in its promoter region (Kulmberg P *et al*, 1992, *Mol. Cell. Biol.*, 12:1932-1939).

The regulation of the three genes, *alcR*, *alcA* and *aldA*, involved in the ethanol utilisation pathway is at the level of transcription (Lockington *et al*, 1987; Gwynne D *et al*, 1987, *Gene*, 51:205-216; Pickett *et al*, 1987, *Gene*, 51:217-226).

There are two other alcohol dehydrogenases present in *A. nidulans*. ADHII is present in mycelia grown in non-induced media and is repressible by the presence of ethanol. ADHII is encoded by *alcB* and is also under the control of *alcR* (Sealy-Lewis & Lockington, 1984). A third alcohol dehydrogenase has also been cloned by complementation with a *adh-* strain of *S cerevisiae*. This gene *alcC*, maps to linkage group VII but is unlinked to *alcA* and *alcR*.

The gene, *alcC*, encodes ADHIII and utilises ethanol extremely weakly (McKnight GL *et al*, 1985, *EMBO J.*, 4:2094-2099). ADHIII has been shown to be involved in the survival of *A. nidulans* during periods of anaerobic stress. The expression of *alcC* is not repressed by the presence of glucose, suggesting that it may not be under the control of *alcR* (Roland LJ and Stromer JN, 1986, *Mol. Cell. Biol.* 6:3368-3372).

In summary, *A. nidulans* expresses the enzyme alcohol dehydrogenase I (ADHI) encoded by the gene *alcA* only when it is grown in the presence of various alcohols and ketones. The induction is relayed through a regulator protein encoded by the *alcR* gene and constitutively expressed. In the presence of inducer (alcohol or ketone), the regulator protein activates the expression of the *alcA* gene. The regulator protein also stimulates expression of itself in the presence of inducer. This means that high levels of the ADHI enzyme are

produced under inducing conditions (i.e. when alcohol or ketone are present). Conversely, the *alcA* gene and its product, ADH1, are not expressed in the absence of inducer. Expression of *alcA* and production of the enzyme is also repressed in the presence of glucose.

Thus the *alcA* gene promoter is an inducible promoter, activated by the *alcR* regulator
5 protein in the presence of inducer (i.e. by the protein/alcohol or protein/ketone combination). The *alcR* and *alcA* genes (including the respective promoters) have been cloned and sequenced (Lockington RA *et al*, 1985, *Gene*, 33:137-149; Felenbok B *et al*, 1988, *Gene*, 73:385-396; Gwynne *et al*, 1987, *Gene*, 51:205-216).

Alcohol dehydrogenase (*adh*) genes have been investigated in certain plant species. In
10 maize and other cereals they are switched on by anaerobic conditions. The promoter region of *adh* genes from maize contains a 300 bp regulatory element necessary for expression under anaerobic conditions. However, no equivalent to the *alcR* regulator protein has been found in any plant. Hence the *alcR/alcA* type of gene regulator system is not known in plants. Constitutive expression of *alcR* in plant cells does not result in the activation of endogenous
15 *adh* activity.

According to a first aspect of the invention, there is provided a chemically-inducible plant gene expression cassette comprising a first promoter operatively linked to a regulator sequence which is derived from the *alcR* gene and encodes a regulator protein, and an
20 inducible promoter operatively linked to a target gene which encodes a protein which is damaging to insects or whose expression induces a metabolic pathway which produces a metabolite which is damaging to insects, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene.

When the target gene encodes an insect-damaging protein, it is advantageous for that
25 protein to be orally active. Examples of orally active insecticidal proteins are *B. thuringiensis* δ endotoxins and therefore, the target gene may encode at least part of a *B. thuringiensis* δ endotoxin.

We have found that the *alcA/alcR* switch is particularly suited to drive genes which encode for *B. thuringiensis* endotoxins for at least the following reasons.

30 The *alcA/alcR* switch has been developed to drive high levels of gene expression. In addition, the regulatory protein *alcR* is preferably driven from a strong constitutive promoter

such as polyubiquitin. High levels of induced transgene expression, comparable to that from a strong constitutive promoter, such as 35 CaMV, can be achieved.

Figure 1 reveals a time course of marker gene expression (CAT) following application of inducing chemical. This study shows a rapid increase (2 hours) of CAT expression following foliar application of inducing chemical. The immediate early kinetics of induction are brought about by expressing the regulatory protein in constitutive manner, therefore no time lag is encountered while synthesis of transcription factors takes place. In addition we have chosen a simple two component system which does not rely on a complex signal transduction system.

We have tested the specificity of *alcA/alcR* system with a range of solvents used in agronomic practice. A hydroponic seedling system revealed that ethanol, butan-2-ol and cyclohexanone all gave high levels of induced reporter gene expression (Figure 2). In contrast when various alcohols and ketones listed in Table 1 and used in agronomic practice were applied as a foliar spray only ethanol gave high levels of induced reporter gene activity (Figure 3). This is of significance since illegitimate induction of transgenes will not be encountered by chance exposure to formulation solvents. Ethanol is not a common component of agrochemical formulations and therefore with appropriate spray management be considered as a specific inducer of the *alcA/alcR* gene switch in a field situation.

Table 1

1. Isobutyl methyl ketone	13. acetonyl acetone
2. Fenchone	14. JF5969 (cyclohexanone)
3. 2-heptanone	15. N-methyl pyrrolidone
4. Di-isobutyl ketone	16. polyethylene glycol
5. 5-methyl-2-hexanone	17. propylene glycol
6. 5-methylpentan-2,4-diol	18. acetophenone
7. ethyl methyl ketone	19. JF4400 (methylcyclohexanone)
8. 2-pentanone	20. propan-2-ol
9. glycerol	21. butan-2-ol
10. γ -butyrolactone	22. acetone
11. diacetone alcohol	23. ethanol
12. tetrahydrofurfuryl alcohol	24. dH ₂ O

A range of biotic and abiotic stresses for example pathogen infection, heat, cold, drought, wounding, flooding have all failed to induce the *alcA/alcR* switch. In addition a range of non-solvent chemical treatments for example salicylic acid, ethylene, absisic acid, auxin, gibberelic acid, various agrochemicals, all failed to induce the *alcA/alcR* system.

5 The present invention is not limited to any particular endotoxin, and is also applicable to chimeric endotoxins.

The first promoter may be constitutive, or tissue-specific, developmentally-programmed or even inducible. The regulator sequence, the *alcR* gene, is obtainable from *Aspergillus nidulans*, and encodes the *alcR* regulator protein.

10 The inducible promoter is preferably the *alcA* gene promoter obtainable from *Aspergillus nidulans* or a "chimeric" promoter derived from the regulatory sequences of the *alcA* promoter and the core promoter region from a gene promoter which operates in plant cells (including any plant gene promoter). The *alcA* promoter or a related "chimeric" promoter is activated by the *alcR* regulator protein when an alcohol or ketone inducer is
15 applied.

The inducible promoter may also be derived from the *aldA* gene promoter, the *alcB* gene promoter or the *alcC* gene promoter obtainable from *Aspergillus nidulans*.

The inducer may be any effective chemical (such as an alcohol or ketone). Suitable chemicals for use with an *alcA/alcR*-derived cassette include those listed by Creaser *et al*
20 (1984, Biochem J, 225, 449-454) such as butan-2-one (ethyl methyl ketone), cyclohexanone, acetone, butan-2-ol, 3-oxobutyric acid, propan-2-ol, ethanol.

The gene expression cassette is responsive to an applied exogenous chemical inducer enabling external activation of expression of the target gene regulated by the cassette. The expression cassette is highly regulated and suitable for general use in plants.

25 The two parts of the expression cassette may be on the same construct or on separate constructs. The first part comprises the regulator cDNA or gene sequence subcloned into an expression vector with a plant-operative promoter driving its expression. The second part comprises at least part of an inducible promoter which controls expression of a downstream target gene. In the presence of a suitable inducer, the regulator protein produced by the first
30 part of the cassette will activate the expression of the target gene by stimulating the inducible promoter in the second part of the cassette.

In practice the construct or constructs comprising the expression cassette of the invention will be inserted into a plant by transformation. Expression of target genes in the construct, being under control of the chemically switchable promoter of the invention, may then be activated by the application of a chemical inducer to the plant.

5 Any transformation method suitable for the target plant or plant cells may be employed, including infection by *Agrobacterium tumefaciens* containing recombinant Ti plasmids, electroporation, microinjection of cells and protoplasts, microprojectile transformation and pollen tube transformation. The transformed cells may then in suitable cases be regenerated into whole plants in which the new nuclear material is stably
10 incorporated into the genome. Both transformed monocot and dicot plants may be obtained in this way.

Examples of genetically modified plants which may be produced include field crops, cereals, fruit and vegetables such as: canola, sunflower, tobacco, sugarbeet, cotton, soya, maize, wheat, barley, rice, sorghum, tomatoes, mangoes, peaches, apples, pears, strawberries,
15 bananas, melons, potatoes, carrot, lettuce, cabbage, onion.

The invention further provides a plant cell containing a gene expression cassette according to the invention. The gene expression cassette may be stably incorporated in the plant's genome by transformation. The invention also provides a plant tissue or a plant comprising such cells, and plants or seeds derived therefrom.

20 The invention further provides a method for controlling plant gene expression comprising transforming a plant cell with a chemically-inducible plant gene expression cassette which has a first promoter operatively linked to a regulator sequence which is derived from the *alcR* gene and encodes a regulator protein, and an inducible promoter operatively linked to a target gene which encodes for a *B. thuringiensis* δ endotoxin, the inducible
25 promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene.

Various preferred features and embodiments of the present invention will now be described by way of the following non-limiting examples and the drawings in which:

30 Figure 1 is a plot showing the time course of induction of AR10 segregating population with 7.5% ethanol;

Figure 2 is a plot showing CAT activity in AR 10-30 homozygous line on root drenching with various chemicals;

Figure 3 is a plot showing CAT activity in AR 10-30 homozygous line on root drenching with various chemicals;

5 Figure 4 shows the production of a 35S regulator construct;

Figure 5 shows the production of a reporter construct;

Figure 6 illustrates switchable insect resistance vectors;

Figure 7 illustrates the sequence of the optimised CryIa(c) gene;

Figure 8 shows the restriction sites in the optimised CryIa(c) gene;

10 Figure 9 illustrates the sequence of the Cry V gene;

Figure 10 shows the vector 5129 bps containing the CryV gene;

Figure 11 illustrates the sequence of the vector pMJB1; and

Figure 12 is a map of vector pJRIi.

EXAMPLE 1

15 **Production Of The alcR Regulator Construct.**

The alcR genomic DNA sequence has been published, enabling isolation of a sample of alcR cDNA.

The alcR cDNA was cloned into the expression vector, pJR1(pUC). pJR1 contains the Cauliflower Mosaic Virus 35S promoter. This promoter is a constitutive plant promoter and will continually express the regulator protein. The nos polyadenylation signal is used in the expression vector.

20 Figure 4 illustrates the production of the 35S regulator construct by ligation of alcR cDNA into pJR1. Partial restriction of the alcR cDNA clone with BamHI was followed by electrophoresis in an agarose gel and the excision and purification of a 2.6 Kb fragment. The fragment was then ligated into the pJR1 vector which had been restricted with BamHI and phosphatased to prevent recircularisation. The alcR gene was thus placed under control of the CaMV 35S promoter and the nos 3' polyadenylation signal in this "35S-alcR" construct.

EXAMPLE 2

30 **Production Of The alcA-CAT Reporter Construct Containing The Chimeric Prom ter.**

The plasmid pCaMVCN contains the bacterial chloramphenicol transferase (CAT) reporter gene between the 35S promoter and the nos transcription terminator (the "35S-CAT" construct).

The alcA promoter was subcloned into the vector pCaMVCN to produce an "alcA-CAT" construct. Fusion of part of the alcA promoter and part of the 35S promoter created a chimeric promoter which allows expression of genes under its control.

Figure 5 illustrates the production of the reporter construct. The alcA promoter and the 35S promoter have identical TATA boxes which were used to link the two promoters together using a recombinant PCR technique: a 246 bp region from the alcA promoter and the 5' end of the CAT gene from pCaMVCN (containing part of the -70 core region of the 35S promoter) were separately amplified and then spliced together using PCR. The recombinant fragment was then restriction digested with BamHI and HindIII. The pCaMVCN vector was partially digested with BamHI and HindIII, then electrophoresed so that the correct fragment could be isolated and ligated to the recombinant fragment.

The ligation mixtures were transformed into E coli and plated onto rich agar media. Plasmid DNA was isolated by miniprep from the resultant colonies and recombinant clones were recovered by size electrophoresis and restriction mapping. The ligation junctions were sequenced to check that the correct recombinants had been recovered.

EXAMPLE 3

Gene Constructs

We have generated the following constructs summarised in Figure 6:

Vector 1 contains the enhanced 35S CaMV promoter fused to the tobacco mosaic virus omega sequence translational enhancer (TMV) *Bacillus thuringiensis* Cry I A (c) gene and nopoline synthase (nos) terminator.

Vector 2 is identical to vector 1 with the exception that the *B. thuringiensis* Cry I A (c) gene is replaced with the *B. thuringiensis* CryV gene.

Vector 3 contains the alc R regulatory protein gene from *Aspergillus nidulans* driven from the 35S CaMV promoter, alc A promoter region, TMV enhancer Cry I A (c) and nos terminator.

Vector 4 is identical to vector 3 with the exception of the Cry I A(c) gene is replaced with the CryV gene.

The Cry I A (c) gene is an optimised Lepidoptera specific synthetic sequence encoding a *Bacillus thuringiensis* endotoxin and is illustrated in Figures 7 and 8. The sequence was obtained from Pamela Green's laboratory, Michigan State University.

The Cry V gene is a novel *Bacillus thuringiensis* endotoxin entomocidal to Coleopteran and Lepidopteran larvae, and is described in our International Patent Publication No WO90/13651. The Cry V gene is a modified synthetic sequence, optimised for plant code usage and has had RNA instability regions removed. It is illustrated in Figures 9 and 10.

EXAMPLE 4

10 **Vector Preparation**

Vector 1 - Constitutive Cry 1A (c)

PCR primers were designed to amplify the TMV omega sequence in pMJB1 (see Figure 9) with the addition of a Sal I site adjacent to the XhoI site (see forward oligonucleotide) and destroying the NcoI site and adding a Sal I and Bgl II sites in the reverse oligonucleotide.

15

Forward oligonucleotide (SEQ ID NO 1)

Sal I

5' CTACTCGAGTCGACTATTTTTACAACAATTACCAAC 3'

XhoI

20

Reverse Oligonucleotide (SEQ ID NO 2)

5' CTAGGTACC GTCGAC GGATCCGTAAGATCTGGTGTAATTGTAAATAGTAATTG 3'

KpnI SalI BamHI BglII

25

A PCR was performed with the forward and reverse primers using pMJB1 plasmid DNA on a template. The resultant PCR product was cloned into the pTag vector (LigATor kit, R&D systems); this was then released with Asp 718 and Xho I digestion and cloned into Xho I/Asp 718 digested pMJB1 (Figure 10), to form pMJB3. pMJB1 is based on pIBT 211 containing the CaMV35 promoter with duplicated enhancer linked to the tobacco mosaic virus translational enhancer sequence replacing the tobacco etch virus 5' non-translated leader, and terminated with the nopaline synthase poly (A) signal (nos).

30

The Cry IA(c) synthetic gene was excised as a Bgl II Bam H I fragment and cloned into pMJB3. A fragment containing the enhanced 35 CaMV promoter TMV omega sequence, CryI A (c) and the nos terminator was isolated using Hind III and EcoR I. The resultant fragment was ligated into EcoRI/Hind III cut pJRli (Figure 12) to generate a Bin 19 based plant transformation vector.

Vector 2 - Constitutive Cry V

pMJB3 was cut with Hind III and a Hind III - EcoRI - Hind III linker was inserted. The resultant vector was then cut with Bam HI and a fragment containing the CryV gene as a Bam HI fragment was inserted. The Cry V gene was orientated using a combination of restriction digestion and sequencing. An EcoRI fragment from the resultant vector, containing the enhanced 35 CaMV promoter, TMV omega sequence, CryV gene and nos terminator, was transferred to JRIRiMCS, a Bin 19 based vector containing the pUC18 multiple cloning site.

Vector 3 - Inducible Cry 1A (c)

pMJB3 containing the Cry 1A(c) gene was cut with Sal I, liberating a fragment containing the TMV omega sequence fused to the Cry 1A(c) gene. The resultant fragment was cloned into Sal I cut palc A CAT and orientated by restriction digest. A fragment containing the alcA promoter fused to the TMV omega sequence, Cry 1A(c) gene and nos terminator was excised using HindIII, and transferred to HindIII digested p35SalcRalcAcat, a Bin 19 based vector containing the 35 CaMV promoter fused to alcR cDNA, with the alcAcat reporter cassette removed on HindIII digestion.

Vector 4 - Inducible Cry V

pMJB3 containing the Cry V gene was cut with Sal I, liberating a fragment containing the TMV omega sequence fused to the Cry V gene. The resultant fragment was cloned into Sal I palcACAT, and orientated by restriction digest and sequence analysis. Two fragments containing the alc A promoter Cry V gene and nos terminator were released by digestion with Hind III. A three way ligation of the two Hind III fragments was performed to insert the alc A Cry V nos cassette into p35alcRalcAcat digested with HindIII to remove the alcAcat cassette. Correct assembly of the cassette was confirmed by restriction digest, southern blotting and sequence analysis.

EXAMPLE 5

Plant transformation

Leaf transformation by *Agrobacterium*.

The transformation was performed according to the method described by Bevan
5 1984. 3-4 weeks old sterile culture of tobacco (*Nicotiana tabacum* cv Samsun), grown on
MS, were used for the transformation. The edges of the leaves were cut off and the leaves
cut into pieces. Then they were put into the transformed *Agrobacterium* cells, containing the
pJR1RI plasmid with the insert, suspension (strain LBA 4404) for 20 minutes. The pieces
were put on plates containing NBM medium (MS medium supplemented with 1mg/l 6-
10 benzylamino purine (6-BAP), 0.1mg/l naphthalene acetic acid (NAA). After 2 days, explants
were transferred to culture pots containing the NBM medium supplemented with carbenicillin
(500 mg/l) and kanamycin (100 mg/l). Five weeks later, 1 shoot per leaf disc was transferred
on NBM medium supplemented with carbenicillin (200 mg/l) and kanamycin (100 mg/l). After
2-3 weeks, shoots with roots were transferred to fresh medium. If required, 2 cuttings from
15 each shoot were transferred to separate pots. One will be kept as a tissue culture stock, the
other one will be transferred to soil for growth in the glasshouse after rooting.

Using this transformation method, the four vectors were introduced into tobacco and
kanamycin-resistant primary transformants generated. There were 53 primary transformants
generated for constitutive Cry1A(c), 54 for constitutive CryV, 73 for inducible Cry1A(c) and
20 62 for inducible CryV.

EXAMPLE 6

Leaf DNA extraction for PCR reactions.

Leaf samples were taken from 3-4 weeks old plants grown in sterile conditions. Leaf
25 discs of about 5 mm in diameter were ground for 30 seconds in 200 ul of extraction buffer
(0.5% sodium dodecyl sulfate (SDS), 250 mM NaCl, 100 mM Tris HCl (tris(hydroxymethyl)
aminomethane hydrochloride), pH 8). The samples were centrifuged for 5 minutes at 13,000
rpm and afterwards 150 ul of isopropanol was added to the same volume of the top layer.

The samples were left on ice for 10 minutes, centrifuged for 10 minutes at 13,000 rpm
30 and left to dry. Then they were resuspended in 100 ul of deionised water. 2.5 ul was used for

the PCR reaction at the conditions described by Jepson *et al* , Plant Molecular Biology Report 9(2), 131-138 (1991).

The primary transgenics generated were tested by PCR analysis to identify plants which contained the full length transgene:

5 Constitutive Cry1A(c)

Two PCR reactions were carried out for these extracts using the following primer pairs:

TMV1 5' CTA CTC GAG TCG ACT ATT TTT ACA ACA ATT ACC AAC
(SEQ ID NO 3)

10 CRY1A2R 5' CGA TGT TGA AGG GCC TGC GGT A (SEQ ID NO 4)

The PCR conditions were 35 cycles of 95 °C 1.2mins, 62 °C 1.8 mins, 72 °C 2.5 mins and extension of 6 mins at 72 °C.

CRY1A1 5' GCA CCT CAT GGA CAT CCT GAA CA (SEQ ID NO 5)

NOS 5' CAT CGC AAG ACC GGC AAC AG (SEQ ID NO 6)

15 The PCR conditions were 35 cycles of 95 °C 0.8 mins, 61 °C 1.8 mins, 72 °C 2.5 mins and extension of 6 mins at 72 °C.

Nine primary transformants gave PCR products for both primer sets; these and two PCR negative lines were planted into soil in 7.5" pots in the glasshouse.

20 Constitutive Cry V

Two PCR reactions were carried out for these extracts using the following primer pairs:

TMV1 (see above)

CryV1R 5' GCT GTA GAT GGT CAC CTG CTC CA (SEQ ID NO 7)

The PCR conditions were 35 cycles of 94 °C 0.8 mins, 64 °C 1.8 mins, 72 °C 2.5 mins and
25 extension of 6 mins at 72 °C.

CRYV1 5' TGT ACA CCG ACG CCA TTG GCA (SEQ ID NO 8)

NOS (see above)

The PCR conditions were 35 cycles of 94 °C 0.8 mins, 58 °C 1.8 mins, 72 °C 2.0 mins and extension of 6 mins at 72 °C.

30 24 primary transformants gave PCR products for both primer sets; these and seven PCR negative lines were planted into soil in 7.5" pots in the glasshouse.

Inducible Cry1A(c)

Three PCR reactions were carried out for these extracts using the following primer pairs:

ALCR1 5' GCG GTA AGG CTT TCA ACA GGC T (SEQ ID NO 9)

NOS as above

- 5 The PCR conditions were 35 cycles of 94 °C 1.0 mins, 60 °C 1.0 mins, 72 °C 1.5 mins and extension of 6 mins at 72 °C.

The primer pairs TMV1/CRY1A2R, CRY1A1/ NOS were used as above.

Forty-five plants gave PCR products for all primer sets; these and two PCR negative lines were planted into soil in 6" pots in the glasshouse

10 Inducible CryV

Sixty-two primary transformants have been generated but no PCR analysis carried out at present.

EXAMPLE 7

15 **Western blot analysis.**

- 120 mg of leaf from 3-4 weeks old plants grown in sterile conditions were ground at 4°C in 0.06 g of polyvinylpoly-pyrrolidone (PVPP) to adsorb phenolic compounds and in 0.5 ml of extraction buffer (1 M Tris HCl, 0.5 M EDTA (ethylenediamine-tetraacetate), 5 mM DTT (dithiothreitol), pH 7.8). Then 200 ml more of extraction buffer were added. The samples
20 were mixed and then centrifuged for 15 minutes at 4°C. The supernatant was removed, the concentration of protein estimated by Bradford assay using the bovine serum albumin (BSA) as standard. The samples were kept at -70°C until required.

- Samples of 25 mg of protein with 33% v/v Laemmli dye (97.5% Laemmli buffer (62.5 mM Tris HCl, 10% w/v sucrose, 2% w/v SDS, pH 6.8), 1.5% pyronin y and 1% b-
25 mercaptoethanol) were loaded on a SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gel (17.7% 30:0.174 acrylamide:bisacrylamide), after 2 minutes boiling.

- Translation products were separated electro-phoretically in the following buffer (14.4% w/v glycine, 1% w/v SDS, 3% w/v Tris Base). Then they were transferred onto nitro-cellulose (Hybond-CÔ, Amersham) using an electroblotting procedure (Biorad unit) in the
30 following blotting buffer (14.4% w/v glycine, 3% w/v Tris Base, 0.2% w/v SDS, 20% v/v methanol) at 40 mV overnight.

- Equal loadings of proteins were checked by staining the freshly blotted nitrocellulose in 0.05% CPTS (copper phthalocyanine tetrasulfonic acid, tetrasodium salt) and 12 mM HCl. Then the blots were destained by 2-3 rinses in 12 mM HCl solution and the excess of dye removed by 0.5 M NaHCO₃ solution for 5-10 minutes followed by rinses in deionised water.
- 5 Filters were blocked for 1 hour with TBS-Tween (2.42% w/v Tris HCl, 8% w/v NaCl, 5% Tween 20 (polyxyethylene sorbitan monolaureate), pH 7.6) containing 5% w/v BSA. Then they were washed for 20 minutes in TBS-Tween supplemented with 2% w/v BSA. Indirect immunodetections were performed with a 1:2000 dilution of a Cry I A (c) or Cry V antiserum as first antibody and with a 1:1000 dilution of a rabbit anti-rabbit antiserum as
- 10 second antibody, associated with the horseradish peroxidase (HRP). Any excess of antiserum was washed with TBS-Tween supplemented with 2% w/v BSA. ECL (enhanced chemiluminescence) detection was performed using the protocols described by Amersham. Any background was eliminated by additional washes of the membranes in the solution mentioned above. The latter one were then subjected to ECL detection.
- 15 An estimation of the level of expression of the *B. thuringiensis* gene was performed on the LKB 2222-020 Ultrosan XL laser densitometer (Pharmacia). A helium-neon laser beam (wavelength 633 nm) was scanning on the autoradiograph a band of 2.4 mm width in the middle of the band corresponding to the translation products.
- Each peak was characterized by its area, determined by the inner software from the curve of
- 20 absorbance function of the beam position.

EXAMPLE 8

Northern blot analysis

- Total RNA was fractionated on a 1.2% agarose gel containing 2.2.M formaldehyde.
- 25 After electrophoresis, the RNA was transferred onto Hybond-N membrane (Amersham) by capillary blotting in 20X SSPE. RNA was fixed to membranes using a combination UV strata linking (Stratagene) and baking for 20 minutes at 80°C. cDNA probes excised from pBluescript SK⁻ by digestion with *Eco*RI, were labelled with a ³²PdCTP using a random priming protocol, described by Feinberg and Vogelstein. Prehybridisations were performed
- 30 in 5X SSPE, 0.1% SDS, 0.1% Marvel (dried milk powder), 100 mg/ml denatured salmon sperm DNA for 4h at 65°C. Hybridizations were achieved in the same buffer containing

labelled probe at 65°C for 12-24h. Filters were washed at 65°C in 3 x SSC 0.1%SDS for 30 mins, and once at 0.5 x SSC 0.1% SDS for 30 mins prior to autoradiography at -80°C.

Insect Feeding trials

- 5 The effectiveness of the present invention can be conveniently tested by feeding leaves of transgenic plants containing the constructs of the present invention to insect larvae, both in the presence and absence, as control, of the inducer.

EXAMPLE 9

10 Primary Screen

- A primary screen was performed by removing leaves from the plants and cutting a number of 1 cm² leaf pieces. Replicas were placed separately on 0.75% agar and each infested with approximately 10 sterilized *Heliothis virescens* eggs. The leaf discs were covered and incubated at 25°C, 70% RH for 5 days before scoring the effects of larval feeding. Leaf damage was assigned a score ranging from 0 to 2 in 0.5 increments; 2 denoting no leaf damage (full insect feeding protection) and 0 implying the leaf disc was fully eaten.

Leaves from all the constitutive Cry1A(c) tissue culture primary transformants and wild type tobacco were removed and tested for effect on *Heliothis virescens* as described above. The results are shown in Table 2 below:

TABLE 2

Replicas:	PCR+/-	A	B	C	D	E
35SCry1A(c) 1		1	1	2	2	2
2		1	1	1.5	1	1.5
3		0	0	0	0	0
4		1.5	1	1.5	0	1.5
5	PCR +	0	1.5	0	1.5	0
6		1.5	0	1	1.5	1.5
7	PCR +	1.5	1.5	1.5	1.5	1.5
8		2	1.5	1	2	2
9	PCR +	2	2	1.5	2	2
10		1.5	2	1	1	1.5
11		0	1.5	0	0	0
12		0	0	0	0	1
13		2	2	0	0	1
14		1.5	1	0	0	0
15		2	1	2	0	0
16	PCR +	2	2	2	2	1.5

Replicas:	PCR+/-	A	B	C	D	E
17		2	0	0	1	2
18		0	0	0	1.5	2
19	PCR +	1.5	1.5	1.5	0	1.5
20	PCR +	1.5	1.5	2	1.5	1.5
21		1	0	0	0	0
22		0.5	0	0	2	2
23		0	1	0	0.5	0.5
24		1.5	1.5	0	0	0
25		2	2	1	1	2
26		1	0	0	1	1
27		0	1.5	0	1.5	0
28	PCR +	1.5	1.5	1.5	1.5	1.5
29		0	0	1	1	1.5
30		1	0	0	1.5	0
31	PCR +	1	0	1.5	0	1.5
32		2	1	1.5	0	1.5
33		2	2	2	2	2
34		1	0	0	1.5	2
35		0	2	0	1	1.5
36		2	0	2	2	0
37		2	1	1.5	1	1.5
38	PCR +	1.5	1.5	1.5	1.5	2
39		1.5	0	0	0	1
40		1	1	0	0	0
41		0	0	1	1	0
42		2	1.5	1.5	1.5	2
43		1.5	1.5	1.5	1.5	2
44		2	1.5	1.5	1.5	2
45		2	2	1	0	0
46		0	2	0	2	1
47		2	2	2	0	0
wt tobacco		1	2	2	2	0

In typical bioassay experiments wild type (wt) tobacco mainly gave an average score of less than 0.5.

5 EXAMPLE 10

Primary Screen - Retest

Eleven of the glasshouse grown constitutive Cry1A(c) plants and wild type tobacco were retested. This was to demonstrate that constitutive Cry1A(c) plants that had been

growing in soil in glasshouse conditions for three weeks after tissue culture were also showing reduced leaf damage from *Heliothis virescens*.

TABLE 3

Identity	a	b	c	d	e
35SCry1A(c) 6	0.5	0.5	2	2	2
7	2	1	2	2	2
9	0.5	0.5	2	1	0.5
16	2	2	2	2	2
19	2	2	1.5	2	1.5
20	1.5	1	0	0.5	2
28	0.5	1.5	2	2	2
31	0	0	0	0	1.5
33	2	2	2	2	2
38	1	0	1	2	1
42	1	1	1	1	1
wt tobacco	0	0	0	0	1.5

5 **EXAMPLE 11**

Primary Screen with CryV Primary Transformants

Leaves from the constitutive CryV primary transformants and wild type tobacco were tested by the method described above. The damage sustained by excised leaf pieces is recorded below in Table 4.

10

TABLE 4

Identity	PCR+/-	a	b	c	d
35SCryV 1	+	0	0	0	0
2		0	0	0	0
3		1.5	0	1	0
4	+	0	0	0	0
5		0	0	0	0
6		0	0	0	0
7	+	0	0	0	0
8	+	0	0	0	0
9	+	0	0	0	0
10	+	0	0	0	0
11	+	0	0	0	0
12		0.5	1	1	0.5
13	+	0	0	0	0
14	+	0	0	0	0
15	+	0	0	0	0

Identity	PCR+/-	a	b	c	d
16		0	0	0	0
17		0	0	0	0
18		0	0	0	0
19		0	0	0	0
20		0	0	0	0
21		0	0	0	0
22	+	0	0	0	0
23	+	0	0	0	0
24	+	0	0	0	0
25	+	0	0	0	0
26	+	0	0	0	0
27		0	0	0	0
28		1	0	1.5	1
29	+	0	0	0	0
30		0	0	0	0
31	+	0.5	0.5	0.5	0
32	+	0	0	0	0
33		0	0	0	0
34		0	1.5	0	0
35		0	0	0	0
36		0	0	0	0
37		0	0	0	1.5
38		0	0	0	0
39		0	0	0	0
40		0	0	0	0
41	+	0	0.5	0	1.5
42		0	0	0	0
43		0	0	0	0
44		0	0	0	0
45	+	0	0	0	0
46	+	0	0	0	0
47		0	0	0	0
88	+	0	0	0	0
49		0.5	0.5	0	0
50		0	0	0	0
51		1	0	0	1
52		1	0.5	0.5	0.5
wt tobacco		0	0	0	1.5

EXAMPLE 12**Secondary Screen**

To verify the data obtained from the primary screen, a secondary assay was performed on transgenic lines on larger leaf pieces using third instar larvae.

- 5 Tobacco leaves were cut from the plant and stored on ice for up to one hour. 40mm diameter leaf discs were cut and placed, cuticle side down, on 3% agar in 50mm plastic pots. Third instar *Heliothis zea* reared on LSU artificial diet for five days at 25°C were weighed and infested onto each leaf disc, one per disc. After infestation lids were placed on the pots and they were stored at 25°C under diffuse light. Treatments were assessed after 3 days for
- 10 mortality, developmental stage and % leaf disc eaten. Larvae were weighed at infestation and after 3 days.

TABLE 5

% LEAF EATEN										
REPLICAS:	A	B	C	D	E	F	G	H	I	J
wt tobacco	20	15	70	20	30	80	30	80	0	95
35SCry1A(c) 7	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
16	<5	<5	<5	<5	10	10	<5	<5	<5	<5
19	<5	10	10	15	10	5	10	<5	<5	20
20	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
28	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
29	20	<5	<5	30	25	25	30	25	25	25
DEVELOPMENTAL STAGE										
wt tobacco	4	3	5	3	3	5	4	5	3	5
35SCry1A(c) 7	3	3	3	3	3	3	3	3	3	3
16	3	3	3	3	3	3	3	3	3	3
19	3	3	3	3	3	3	3	3	3	4
20	3	3	3	3	3	3	3	3	3	3
28	3	3	3	3	3	3	3	3	3	3
29	4	3	3	5	4	4	4	4	3	3
MORTALITY										
wt tobacco	L	L	L	L	L	L	L	L	D	L
35SCry1A(c) 7	L	D	D	L	D	D	D	D	D	D
16	D	D	D	D	L	L	L	L	D	L
19	D	L	L	L	L	L	L	D	L	L
20	D	D	D	D	D	D	L	L	D	L
28	D	L	D	D	D	D	D	L	L	D
29	L	L	D	L	L	L	L	L	L	L

EXAMPLE 13**Inducible insecticidal activity**

Forty -five inducible Cry1A(c) PCR positive lines, two PCR negative lines and wt tobacco in 6" pots were root drenched with 100mls of 5% ethanol. 28 hours later 4 replica
 5 small leaf pieces were removed and infested with *Heliothis virescens* eggs. The results are shown below (Table 6). Of the 45 lines grown in the presence of ethanol, 66% showed full resistance on the primary screen test to *Heliothis virescens*. To demonstrate that the plants were inducible and not constitutive expressors leaves were removed 8 days later from 7 of
 10 the high scoring lines and infested with *Heliothis virescens* eggs. Previous data from a reporter gene driven by the 35SalcRalcA switch promoter showed that CAT protein levels peaked at 24/48 hours and was on the decline after 48 hours (Figure 1). Other data (not shown) demonstrated that no CAT protein was detected 9 days after induction.

Table 7 demonstrates that in the absence of ethanol irrigation mortality levels were found to be comparable to that seen with a wild type control.

15

TABLE 6

<i>Heliothis virescens</i> on ALC Cry1A(c)					
glasshouse primary transgenics.					
5% ethanol root drench, ~28hours before assay set up.					
identity	PCR+/-	a	b	c	d
ALCCry1A(c) 1	+	1	2	2	2
2	+	2	2	2	2
3	+	0	0	0	1.5
4	+	2	2	2	2
5	+	0.5	0.5	0.5	1
6	-	2	1	0.5	0
7	+	0	2	1.5	2
8	+	2	2	2	2
9	+	2	2	2	2
10	+	2	2	2	2
11	+	2	2	2	2
12	+	2	2	2	2
13	+	2	2	2	2
14	+	2	2	2	2
15	+	2	2	2	2
16	+	2	2	2	2
17	+	2	2	2	2

18	+	2	2	2	2
19	+	2	2	2	2
20	+	2	2	2	2
21	+	0	0	0	1
22	+	0.5	2	0.5	2
23	+	2	2	2	2
24	+	2	2	2	2
25	+	2	2	2	2
26	+	2	2	2	2
27	+	1	2	2	2
28	+	0.5	2	2	2
29	+	2	2	2	2
30	+	2	2	2	2
31	+	2	2	1	2
32	+	2	2	2	2
33	+	1	2	2	2
34	+	2	2	2	2
35	+	1	2	2	2
36	+	2	2	2	1
37	+	0	0.5	0	1
38	-	0.5	0.5	0.5	0.5
39	+	2	2	2	2
40	+	2	2	2	2
41	+	2	2	2	2
42	+	2	2	2	2
43	+	2	2	2	2
44	+	2	2	2	2
45	+	2	2	2	0.5
46	+	2	2	2	2
47	+	1	2	2	2
wt tobacco		0	0.5	0	0.5

TABLE 7

INDUCED						NO INDUCTION					
identity	PCR+/-	a	b	c	d	identity	PCR+/-	a	b	c	d
17	+	2	2	2	2	17	+	0.5	0.5	0.5	1
32	+	2	2	2	2	32	+	1.5	1	1	0.5
39	+	2	2	2	2	39	+	2	2	0.5	0.5
40	+	2	2	2	2	40	+	0.5	0.5	0.5	0.5
41	+	2	2	2	2	41	+	0.5	2	0	1
43	+	2	2	2	2	43	+	0.5	0.5	0.5	0.5
44	+	2	2	2	2	44	+	0.5	0	0.5	0.5
wt tobacco		0	0.5	0	0.5	wt tobacco		0	0	0.5	0.5
						wt tobacco		0	1	0	0

Several lines were chosen for a secondary screen to test the effect of induction on insect feeding, along with the constitutive Cry1A(c) line 10 and wt tobacco as controls. 10 leaf pieces for each line were removed from primary transformants 12 days after they had been induced by root drenching with 100mls of 5% ethanol and placed on 3% agar in 50 mm pots with lids and incubated overnight at 25°C and 60% humidity. Expression of Cry1A(c) protein was expected to be low or undetectable after 12 days.. The plants were then root drenched with 100mls of 5% ethanol. 22 hours later leaves were excised and ten 40mm leaf pieces were removed and placed on 3% agar in 50mm pots with lids. Five uninduced and 5 ethanol induced leaf discs were infested with 3rd instar *Heliothis zea* and 5 of each infested with *Heliothis virescens* reared as described above. Table 8 demonstrates that wild type controls in the presence or absence of ethanol show a high percentage leaf disc eaten, while the 35S controls show good insect control under both chemical regimes. Transgenic lines containing the Alc Cry IA(c) construct showed poor insect control in the absence of ethanol treatment. Table 8 shows induction with ethanol gives insect control comparable to that seen in the 35S Cry I A (c) control.

TABLE 8

nos 1-5= <i>H. zea</i>						
nos 6-10= <i>H. virescens</i>						
line		%eaten		%eaten		
wt uninduced	1	45		wt induced	1	55
	2	0			2	55
	3	25			3	95
	4	30			4	50
	5	45			5	25
	6	95			6	25
	7	5			7	55
	8	50			8	30
	9	20			9	20
10	15		10	25		
35S/10 uninduced	1	10		35S/10 induced	1	<5
	2	<5			2	<5
	3	15			3	5
	4	10			4	5

	5	<5			5	5
	6	0			6	<5
	7	<5			7	10
	8	<5			8	<5
	9	<5			9	0
	10	<5			10	5
66 uninduced	1	15		66 induced	1	<5
	2	10			2	<5
	3	0			3	<5
	4	50			4	<5
	5	50			5	10
	6	10			6	0
	7	10			7	<5
	8	15			8	<5
	9	0			9	<5
	10	10			10	<5

- 25 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT:
(A) NAME: ZENECA LIMITED
(B) STREET: 15 Stanhope Gate
(C) CITY: London
10 (E) COUNTRY: UK
(F) POSTAL CODE (ZIP): W1Y 6LN
- (ii) TITLE OF INVENTION: DNA CONSTRUCTS
- 15 (iii) NUMBER OF SEQUENCES: 9
- (iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
20 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)
- (vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9516241.8
25 (B) FILING DATE: 08-AUG-1995

(2) INFORMATION FOR SEQ ID NO: 1:

- 30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
35 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CATCTCGAGT CGACTATTTT TACAACAATT ACCAAC

36

(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
50 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CTAGGTACCG TCGACGGATC CGTAAGATCT GGTGTAATTG TAAATAGTAA TTG

53

(2) INFORMATION FOR SEQ ID NO: 3:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

15 CTACTCGAGT CGACTATTTT TACAACAATT ACCAAC

36

(2) INFORMATION FOR SEQ ID NO: 4:

- 20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: DNA

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CGATGTTGAA GGGCCTGCGG TA

22

(2) INFORMATION FOR SEQ ID NO: 5:

- 35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
40 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCACCTCATG GACATCCTGA ACA

23

50 (2) INFORMATION FOR SEQ ID NO: 6:

- 55 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

60

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CATCGCAAGA CCGGCAACAG

20

(2) INFORMATION FOR SEQ ID NO: 7:

5

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

10

- (ii) MOLECULE TYPE: DNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GCTGTAGATG GTCACCTGCT CCA

20

23

(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

25

- (ii) MOLECULE TYPE: DNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TGTACACCGA CGCCATTGGC A

35

21

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

40

45

- (ii) MOLECULE TYPE: DNA

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

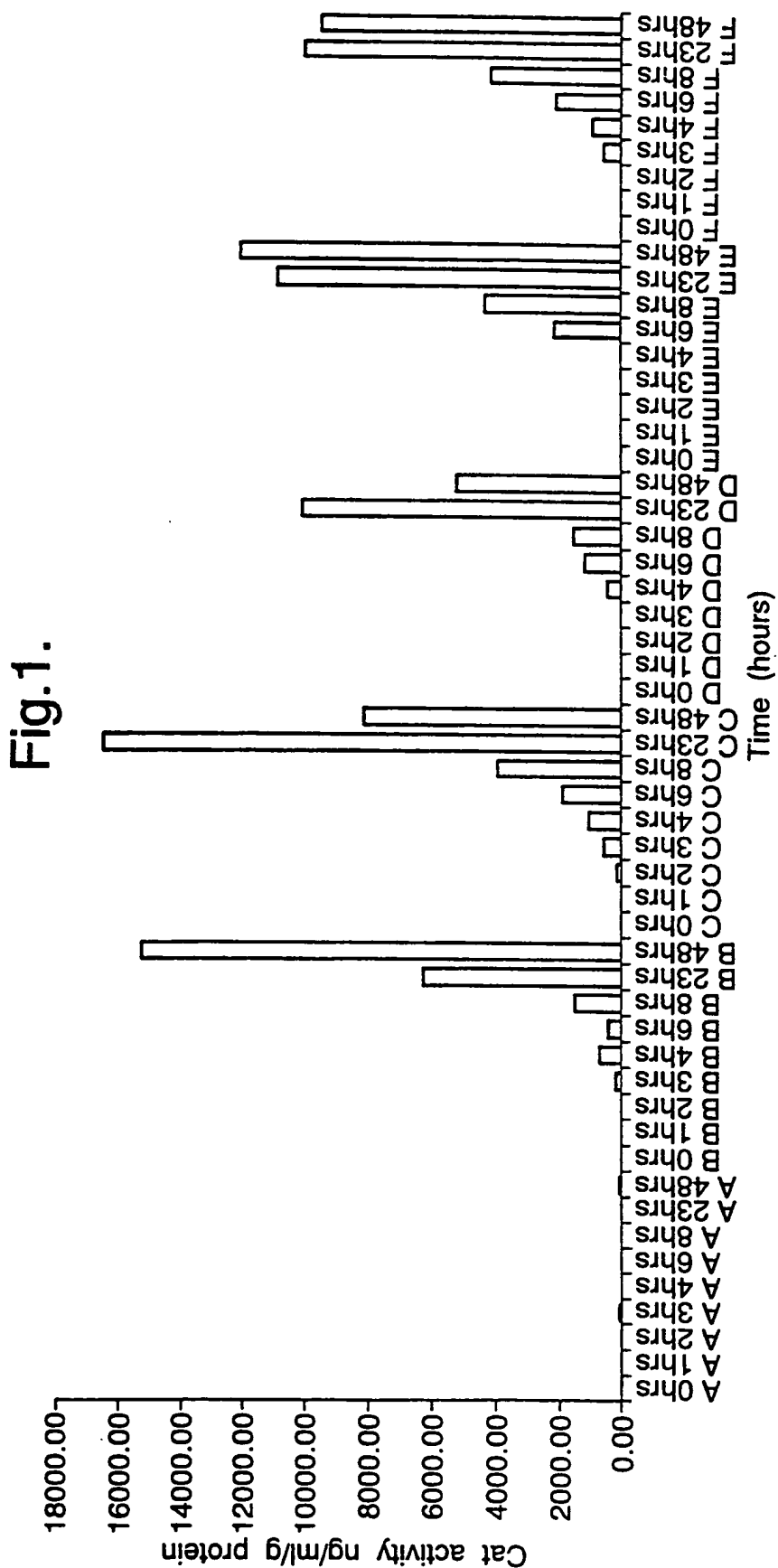
GCGGTAAGGC TTTCAACAGG CT

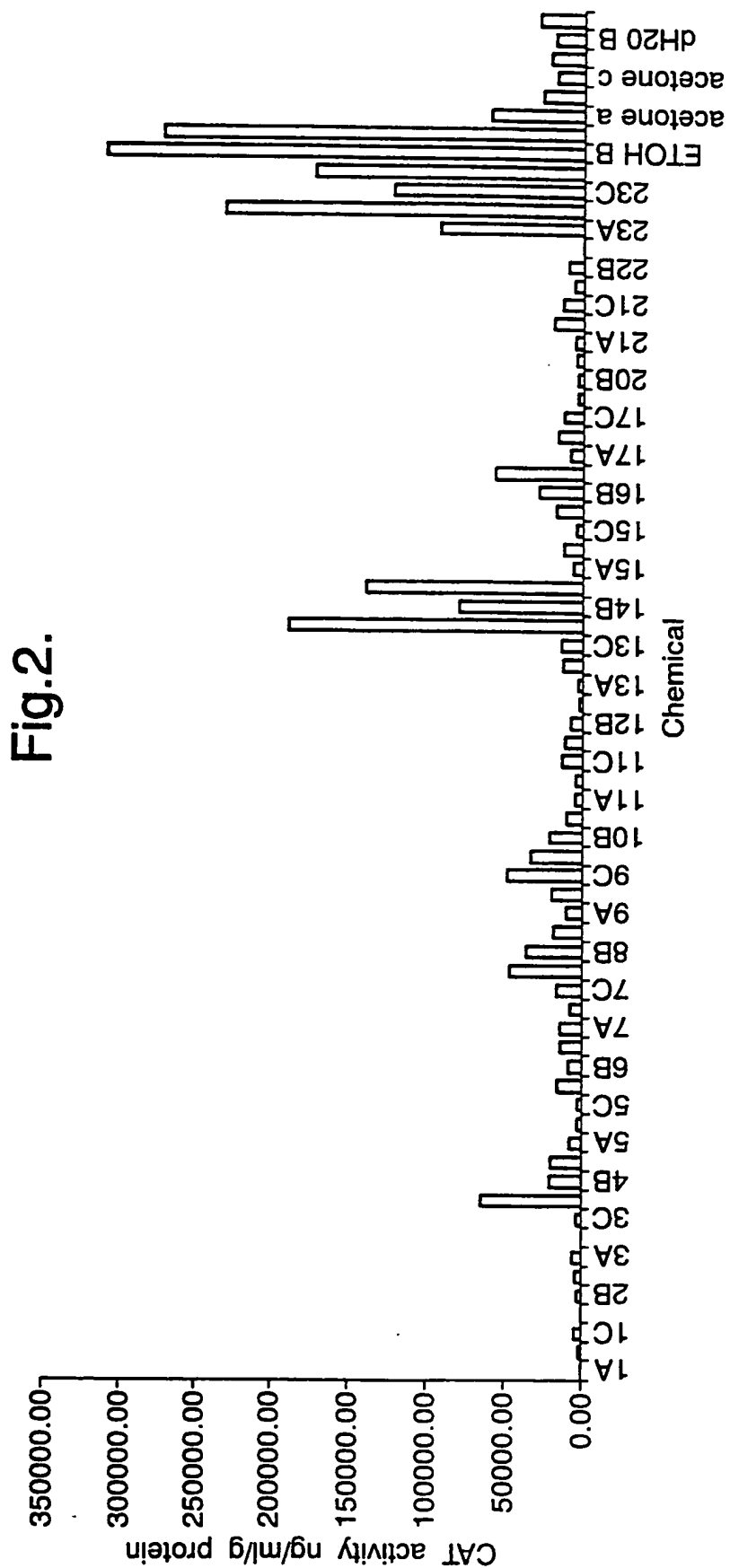
22

CLAIMS

1. A chemically-inducible plant gene expression cassette comprising a first promoter operatively linked to a regulator sequence which is derived from the *alcR* gene and encodes a
5 regulator protein, and an inducible promoter operatively linked to a target gene which encodes a protein which is damaging to insects or whose expression induces a metabolic pathway which produces a metabolite which is damaging to insects, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene.
10
2. A chemically inducible plant gene expression cassette as claimed in claim 1, wherein the target gene encodes an orally active insecticidal protein.
3. A chemically inducible plant gene expression cassette as claimed in claim 2, wherein
15 the orally active insecticidal protein is at least part of a *Bacillus thuringiensis* δ endotoxin.
4. A plant gene expression cassette according to any one of claims 1 to 3, wherein the inducible promoter is derived from the *alcA* gene promoter.
- 20 5. A plant gene expression cassette according to any one of claims 1 to 4, wherein the inducible promoter is a chimeric promoter.
6. A plant cell containing a plant gene expression cassette according to any preceding claim.
25
7. A plant cell according to claim 6, wherein the plant gene expression cassette is stably incorporated in the plant's genome.
8. A plant tissue comprising a plant cell according to either of claims 6 and 7.
- 30 9. A plant comprising a plant cell according to either of claims 6 and 7.

10. A plant derived from a plant according to claim 9.
11. A seed derived from a plant according to either of claims 9 and 10.
- 5 12. A method of controlling insects comprising transforming a plant cell with the plant gene expression cassette of any one of claims 1 to 5.





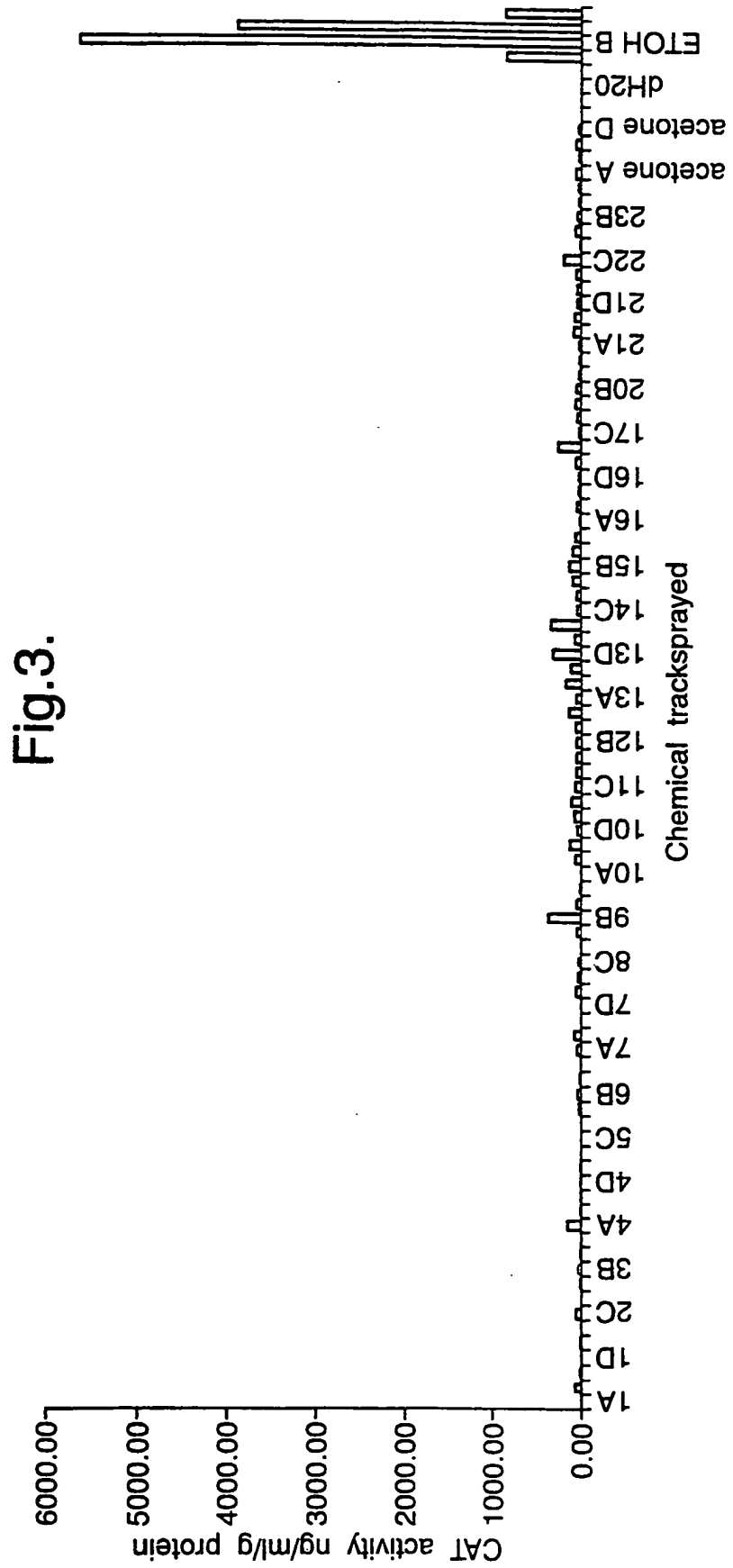


Fig. 4(1/2).

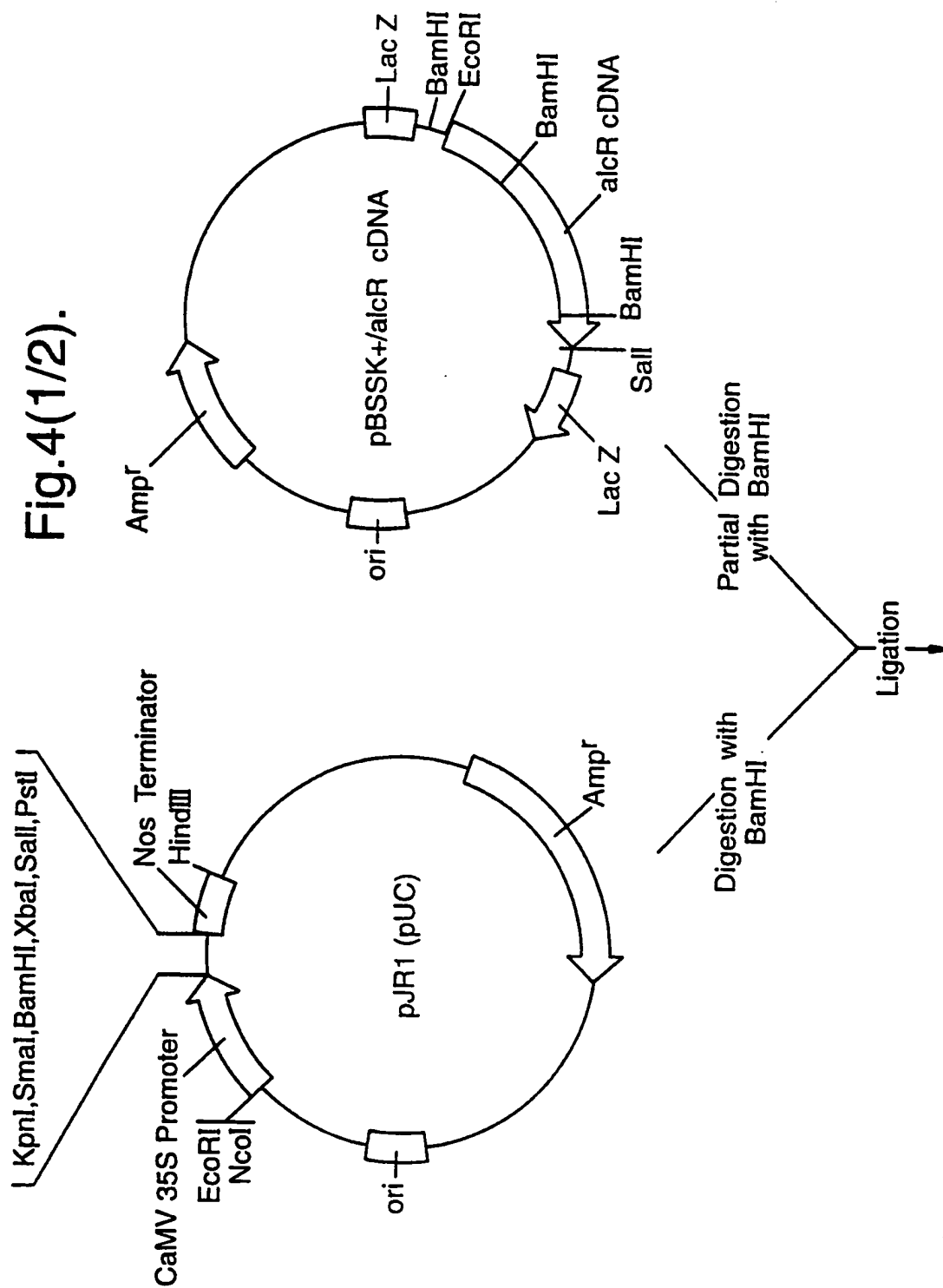


Fig.4(2/2).

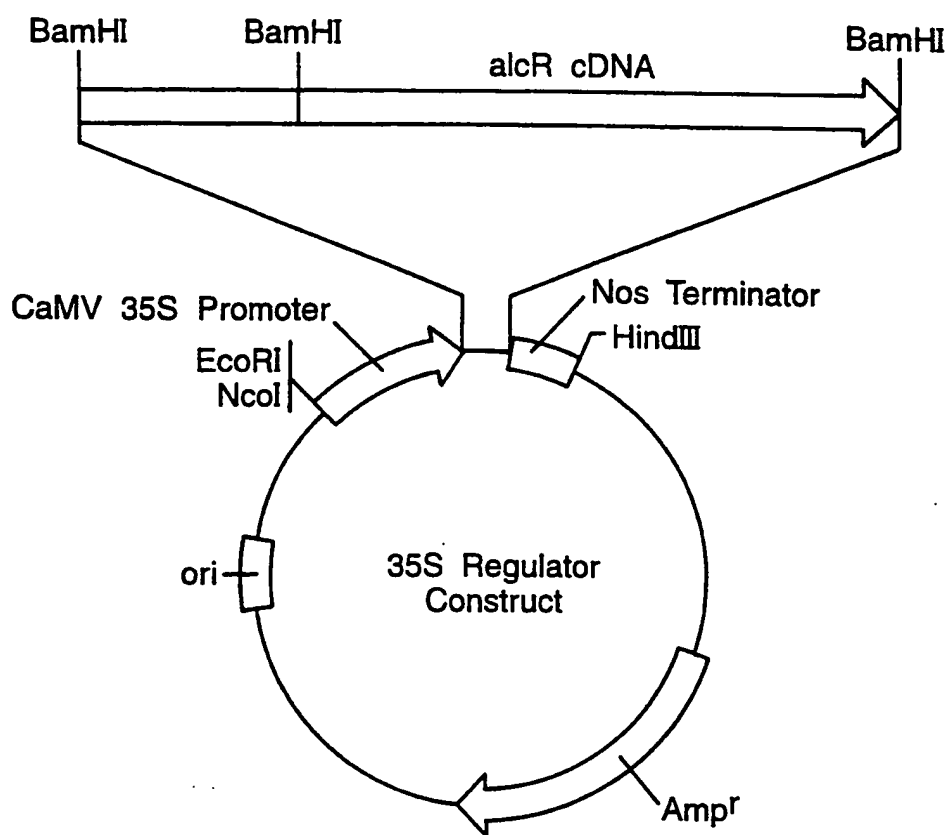


Fig.5(1/3).

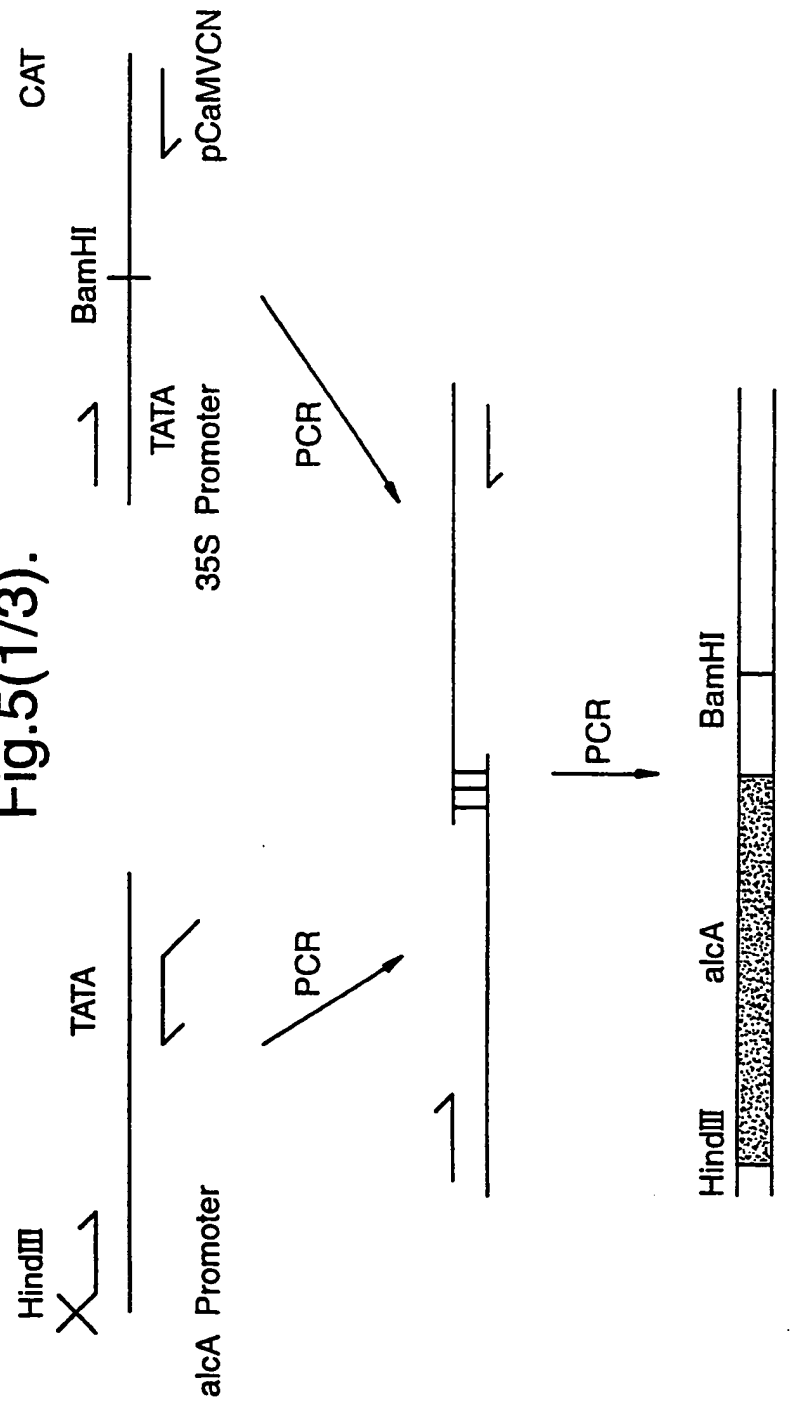


Fig.5(2/3).

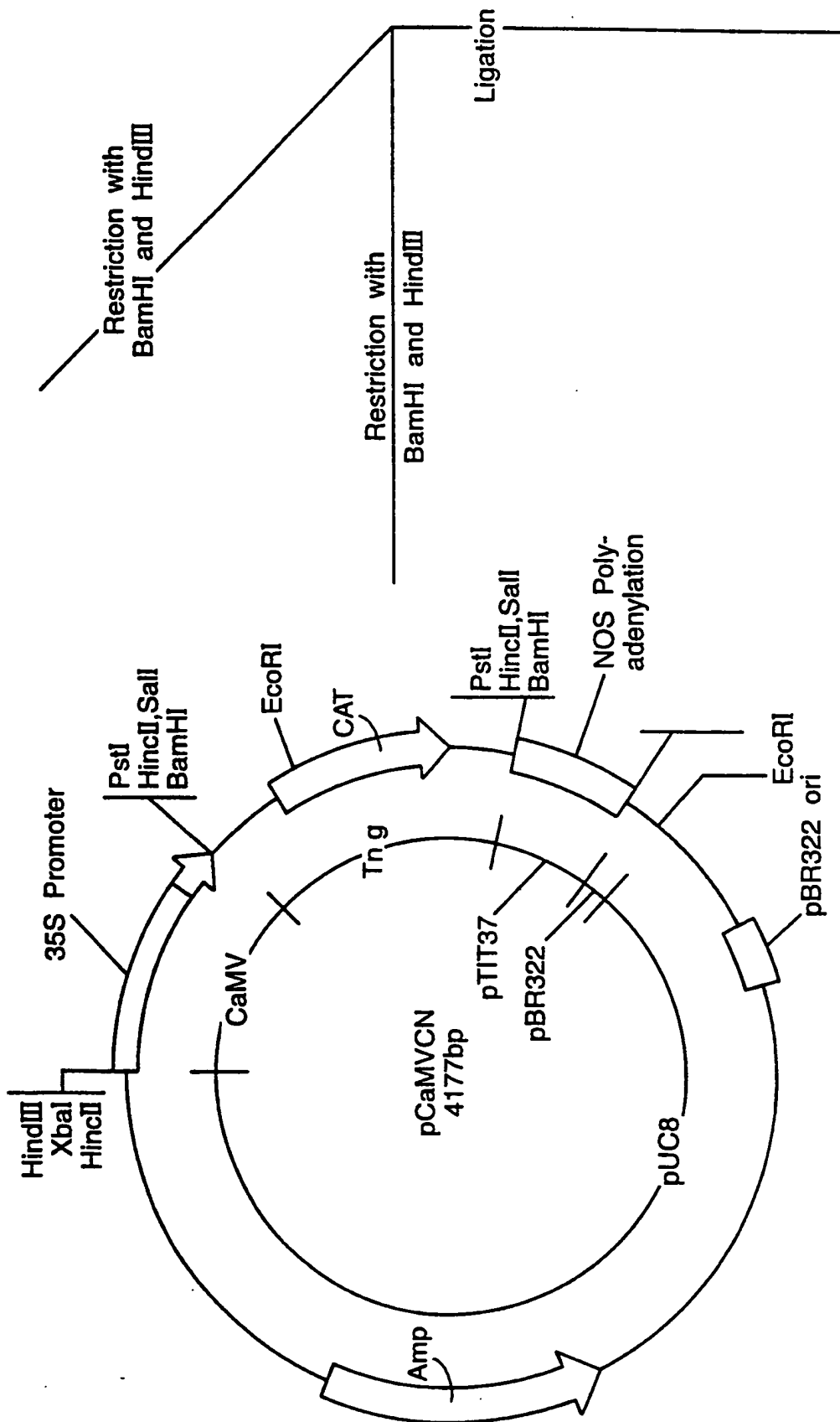


Fig.5(3/3).

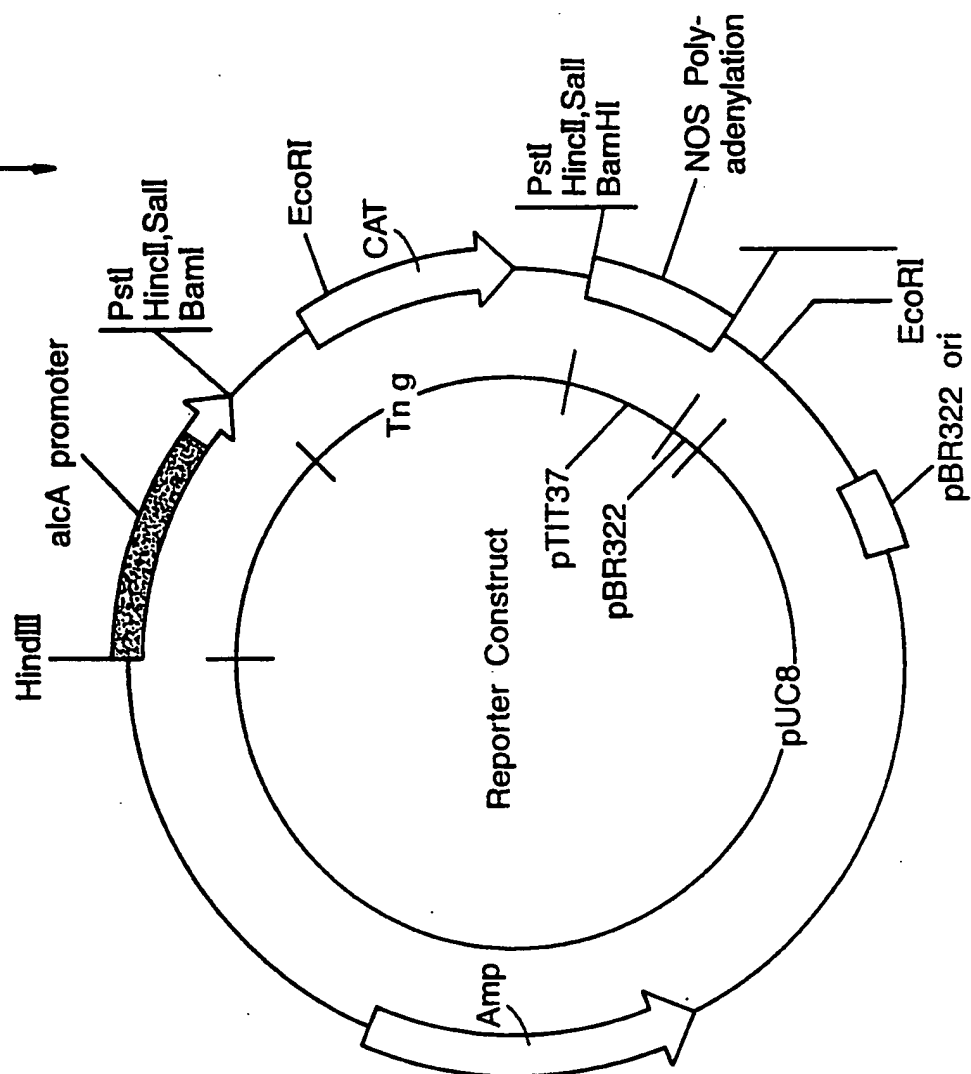
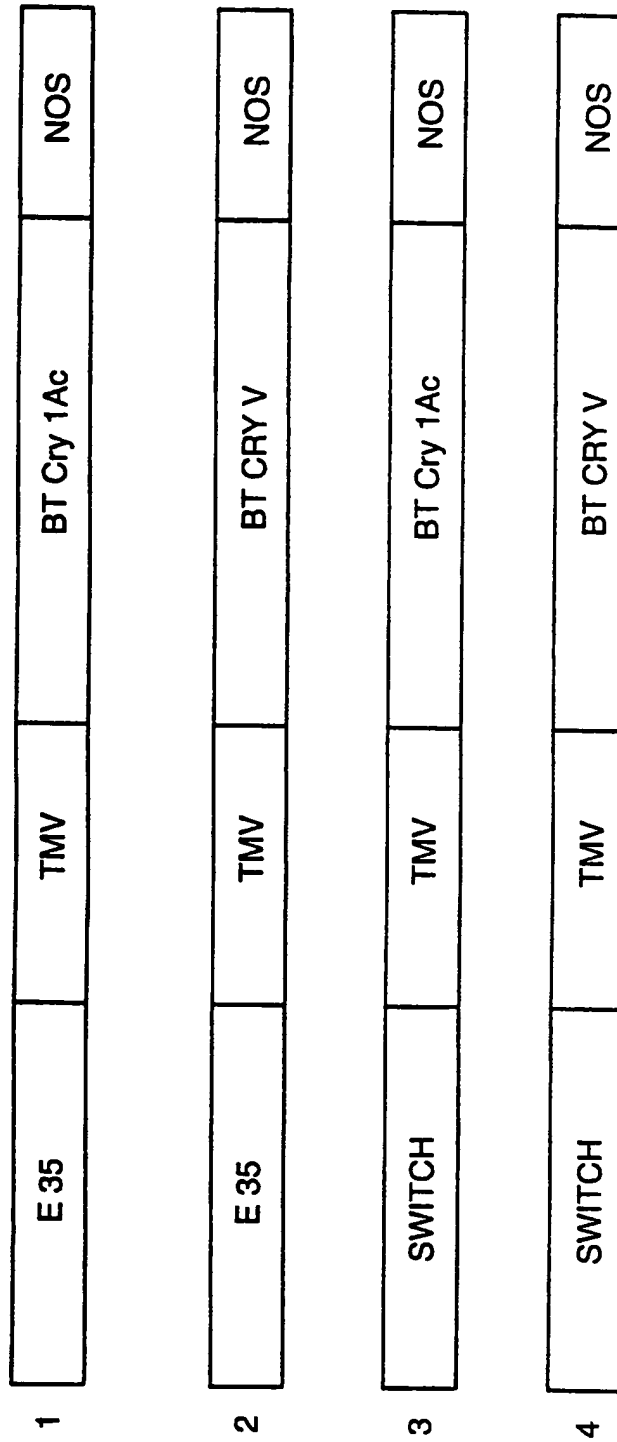


Fig.6.



E 35 = ENHANCED CaMV35

TMV = TMV ENHANCER

SWITCH - 35S alc R alc A promoter

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Fig.7.

1	AGATCTTAA	CAATGCTAT	GATTACGCCT	AGCTTGCAATG	CCTGCATCCC	GTACAACTGC
	TCTAGAATTT	GTTACCGATA	CTAATGCGGA	TCGAACGTAC	GGACGTAGGG	CATGTTGACG
61	CTGAGCAACC	CGGAGGTGGA	GGTGCTGGGC	GGCGAGCGCA	TCGAGACCCG	CTACACGCCG
	GACTCGTTGG	GCCTCCACCT	CCACGACCCG	CCGCTCGCGT	AGCTCTGGCC	GATGTGCGGC
121	ATCGACATCT	CCCTGTCCCT	CACCCAGTTC	CTGCTGAGCG	AGTTCGTCCC	GGCGCCCGGC
	TAGCTGTAGA	GGGACAGGA	GTGGGTCAAG	GACGACTCGC	TCAAGCAGGG	CCCGCGCGCC
181	TTCGTGCTGG	GCCTGGTGGA	CATCATCTGG	GGCATCTTCG	GCCCCTCCCA	GTGGGACGCC
	AAGCACGACC	CGGACCACCT	GTAGTAGACC	CCGTAGAAGC	CGGGCAGGGT	CACCCCTGCGG
241	TTCCTGGTGC	AGATCGAGCA	GCTGATCAAC	CAGCGCATCG	AGGAGTTCGC	CCGCAACCAG
	AAGGACCACG	TCTAGCTCGT	CGACTAGTTG	GTCGCGTAGC	TCCCTCAAGCG	GGCGTTGGTC
301	GCCATCTCTA	GA CTGGAGGG	CCTGAGCAAC	CTGTACCATA	TCTACGCCGA	GTCCTTCCGC
	CGGTAGAGAT	CTGACCTCCC	GGACTCGTTG	GACATGGTTT	AGATGCGGCT	CAGGAAGGCG
361	GAGTGGGAGG	CTGACCCGAC	CAACCCGGCC	CTGAGGGAGG	AGATGGGCAT	CCAGTTCAAC
	CTCACCCCTCC	GACTGGGCTG	GTTGGGCCCG	GACTCCCTCC	TCTACGCCGA	GGTCAAGTTG
421	GACATGAACT	CCGCCCTGAC	CACCGCCATC	CCGCTGTTTC	CCGTGCAGAA	CTACCAAGTG
	CTGTACTTGA	GGCGGGACTG	GTGGCGGTAG	GGCGACAAGC	GGCAGCTCTT	GATGGTCCAC
481	CCGCTCCTGT	CCGTGTACGT	GCAGGCCGCC	AACCTCCACC	TGTCGGTCTT	GAGGGACGTG
	GGCGAGGACA	GGCACATGCA	CGTCCGGCCG	TTGGAGGTGG	ACAGCCAGGA	CTCCCTGCAC

1 1 / 2 2

Fig.7 (Cont).

81 CAGGGCGTGT ACAGGACCCT CTCCTCCACC CTGTACCGCA GGGCCCTTCAA CATCGGCATC
 GTCCCGCACA TGTCCCTGGGA GAGGAGGTGG GACATGGCGT CCGGGAAGTT GTAGCCGTAG

 141 AACAAACCAGC AGCTGTCCGT CCTGGACGGC ACCGAGTTCTG CCTACGGCAC CTCCTCCAAC
 TTGTTGGTCTG TCGACAGGCA GGACCTGCCG TGGCTCAAGC GGATGCCGTG GAGGAGGTTG

 1201 CTGCCCCTCCG CCGTATACAG GAAGAGCGGC ACCGTGGACT CCCTGGACGA GATCCCGCCG
 GACGGGAGGC GGCATATGTC CTTCTCGCCG TGGCACCTGA GGGACCTGCT CTAGGGCGGC

 1261 CAGAACAAACA ACGTCCC GCCG GAGGCAGGGC TTCAGCCACC GCCTGAGCCA CGTGTCCTATG
 GTCTTGTTGT TGCAGGGCGG CTCCGTCCCC AAGTCGGTGG CGGACTCGGT GCACAGGTAC

 1321 TTCCGCTCCG GCTTCAGCAA CAGCAGCGTG AGCATCATCA GGGCCCCCGAT GTTCTCCTGG
 AAGCGAGGC CGAAGTCGTT GTCGTCGCAC TCGTAGTAGT CCGGGGGCTA CAAGAGGACC

 1381 ATTCAACCGCA GCGCCGAGTT CAACAACATC ATCGCCTCCG ACAGCATCAC CCAGATCCCG
 TAAGTGGCGT CGCGGCTCAA GTTGTGTAG TAGCGGAGGC TGTCGTAGTG GGTCTAGGGC

 1441 GCTGTGAAGG GCAACTTCCT GTTCAACGGC TCCGTGATCT CCGGCCCGGG GTTCACCCGGC
 CGACACTTCC CGTTGAAGGA CAAGTTGCCG AGGCACTAGA GGCCGGGGCC GAAGTGGGCC

 1501 GGCAGCCTCG TCAGGCTGAA CAGCTCCGGC AACAAACATCC AGAACCGCGG CTACATCGAG
 CCGCTGGAGC AGTCCGACTT GTCGAGGGCCG TTGTTGTAGG TCTTGGCGCC GATGTAGCTC

Fig.7 i.

```

541 TCCGTGTTCC GCCAGCGCTG GGGCTTCGAC GCCGCCACCA TCAACAGCCG CTACAACGAC
    AGGCACAAGC CGGTCGCGAC CCCGAAGCTG CGGCGGTGGT AGTTGTCTGGC GATGTTGCTG

601 CTGACCAAGC TGATCGGCAA CTACACCGAC TACGCCGTCC GCTGGTACAA CACCGGCCTG
    GACTGGTCCG ACTAGCCGTT GATGTGGCTG ATGCGGCAGG CGACCATGTT GTGGCCGGAC

661 GAGCGCGTGT GGGGCCCGGA CTCTAGAGAC TGGGTCAGGT ACAACCAAGTT CAGGCGCGAG
    CTCGGCGACA CCCCAGGCTT GAGATCTCTG ACCCAGTCCA TGTTGGTCAA GTCCGCGCTC

721 CTCACCTCA CCGTGCTGGA CATCGTGGCC CTGTTCCCCA ACTACGACTC CAGGAGGTAC
    GAGTGGGAGT GGCACGACCT GTAGCACCGG GACAAGGSET TGATGCTGAG GTCCCTCCATG

781 CCCATCAGGA CCGTGAGCCA GCTGACCAGG GAAATCTACA CCAACCCCGT GCTGGAGAAC
    GGGTAGTCCT GGCACCTCGT CGACTGGTCC CTTTAGATGT GGTTGGGGCA CGACCTCTTG

841 TTCGACGGCA GCTTCCGCGG CAGCGCCCGG GGCATCGAGA GGAGCATCAG GAGCCCGCAC
    AAGCTGCCGT CGAAGGCGCC GTCGCGGGTC CCGTAGCTCT CCTCGTAGTC CTCGGGCGTG

901 CTCATGGACA TCCTGAACAG CATCACCATC TACACCGACG CCCACCGCGG CTACTACTAC
    GAGTACCTGT AGGACTTGTC GTAGTGGTAG ATGTGGCTGC GGTGGCGCC GATGATGATG

961 TGGTCCGGCC ACCAGATCAT GGCCTCCCCC GTGGGCTTCT CCGGCCCGGA GTTACCTTTC
    ACCAGGCCCG TGGTCTAGTA CCGGAGGGGG CACCCGAAGA GGCCGGGCTT CAAGTGAAG

1021 CCGCTGTACG GCACCATGGG CAACGCGCGC CCGCAGCAGA GGATCGTCGC CCAGCTCGGC
    GCGGACATGC CGTGGTACCC GTTGGGCGGG GCGTCTGTCT CCTAGCAGCG GTCGAGCCG

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1 3 / 2 2

Fig.7 (Cont i).

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61  GTCCCCGATCC ACTTCCCGTC CACCTCCACC AGGTACAGGG TGAGGGTCCG CTACGGCCTCC
    CAGGGCTAGG TGAAGGGCAG GTGGAGGTGG TCCATGTCCC ACTCCAGGC GATGCGGAGG

1621 GTGACCCCGA TCCACCTCAA CGTGAACCTGG GGCAACTCCT CCATCTTCTC CAACACCGTC
    CACTGGGCT AGGTGGAGTT GCACTTGACC CCGTTGAGGA GGTAGAAGAG GTTGTGGCAG

1681 CCGGCCACCG CCACCTCCCT CGACAACCTC CAGTCCAGCG ACTTCGGCTA CTTCGAGAGC
    GGCCGGTGGC GTGGAGGGA GCTGTTGGAG GTCAGGTCGC TGAAGCCGAT GAAGCTCTCG

1741 GCCAACGCCT TCACCTCCTC CCTCGGCAAC ATCGTCGGCG TCAGGAACTT CTCCGGCACC
    CCGTTGCCGA AGTGGAGGAG GGAGCCGTTG TAGCAGCCGC AGTCCTTGAA GAGGCCGTGG

1801 GCTGGCGTGA TCATCGACAG GTTCGAGTTC ATCCCGGTCA CCGCCACCCT CGAGCCGCGC
    CGACCGCACT AGTAGCTGTC CAAGCTCAAG TAGGGCCAGT GGCGGTGGGA GCTCGGCGGC

1861 TAGGATCC
    ATCCTAGG

```

Tot number of bases is: 1868.
 DNA sequence composition: 361 A; 702 C; 491 G; 314 T;

Sequence name: NCRY1AC.

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1 4 / 2 2

Fig.8.

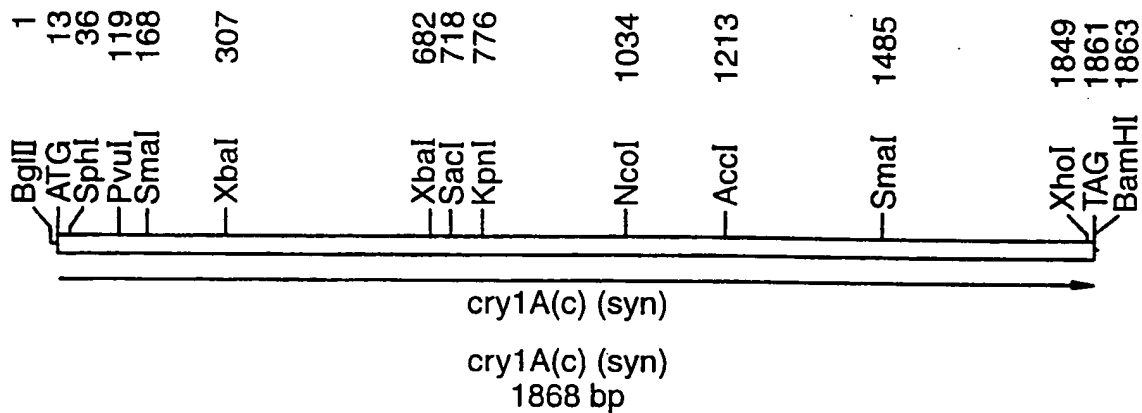
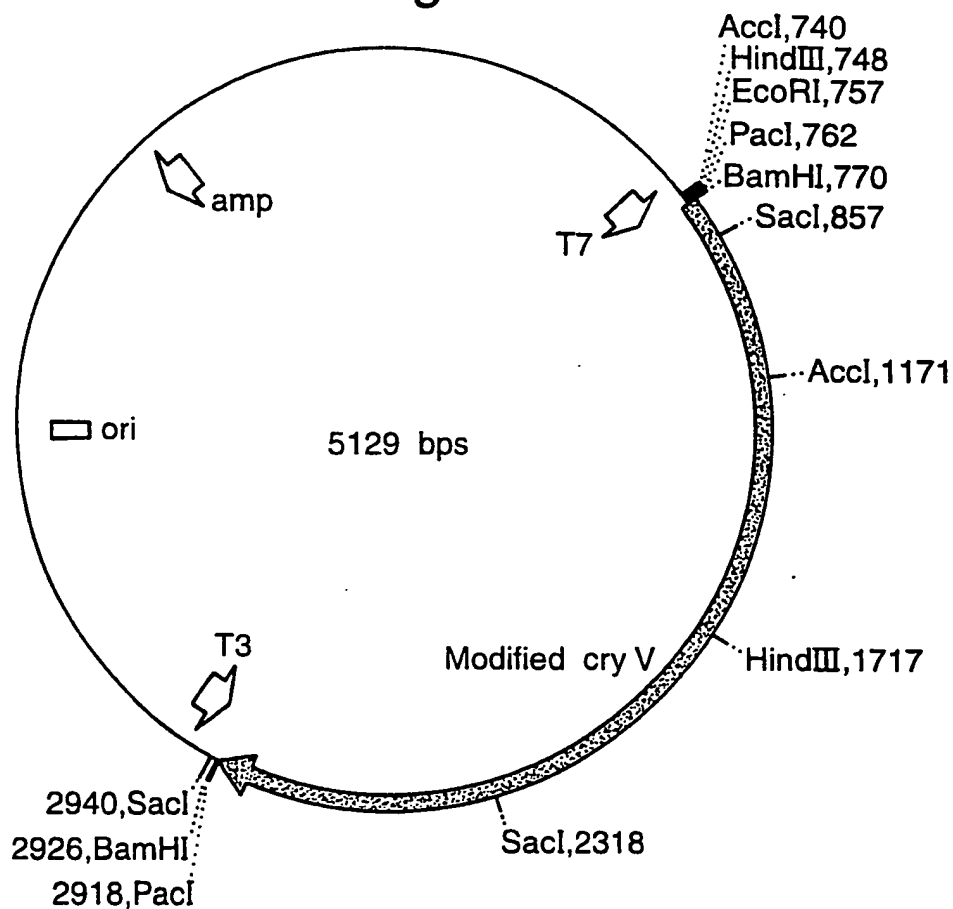


Fig.10.



The second version of the modified cry V gene
in pBluescript (sk). NBI

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Fig.9.

1	ATGAAGCTGA	AGAACCAAGA	CAAGCACCAA	TCGTTCTCCA	GCAACGCGAA	AGTGGACAAG
	TACTTCGACT.	TCTTGGTTCT	GTTCGTGGTT	AGCAAGAGGT	CGTTGCGCTT	TCACCTGTTT
61	ATCAGCACCG	ACTCCCTGAA	GAACGAGACC	GACATCGAGC	TCCAGAAACAT	CAACCACGAA
	TAGTCGTGGC	TGAGGGACTT	CTTGCTCTGG	CTGTAGCTCG	AGGTCTTGTA	GTTGGTGTCT
121	GATTGCCCTGA	AGATGTCCGA	GTACGAGAAC	GTGGAGCCGT	TCGTGAGCGC	CTCCACCATC
	CTAACGGACT	TCTACAGGCT	CATGCTCTTG	CACCTCGGCA	AGCACTCGCG	GAGGTGGTAG
181	CAGACCGGCA	TCGGCATCGC	GGGCAAGATC	CTGGGTACCC	TGGGCGTGCC	GTTTGCCGGC
	GTCTGGCCGT	AGCCGTAGCG	CCCGTTCTAG	GACCCATGGG	ACCCGCACGG	CAAACGGCCG
241	CAAGTGGCTA	GCCTGTACAG	CTTCATCCTC	GGCGAGCTGT	GGCCTAAGGG	CAAGAACCAA
	GTTCAACCGAT	CGGACATGTC	GAAGTAGGAG	CCGCTCGACA	CCGGATTCCC	GTTCTTGGTT
301	TGGGAGATCT	TCATGGAGCA	CGTGGAGGAG	ATCATCAACC	AGAAGATTTC	CACCTACGCC
	ACCCCTCTAGA	AGTACCCTCGT	GCACCTCCTC	TAGTAGTTGG	TCTTCTAAAG	GTGGATGCGG
361	CGCAACAAGG	CCCTTACCGA	CCTGAAGGGC	CTCGGCGACG	CCCTGGCTGT	CTACCACGAC
	GCGTTGTTCC	GGGAATGGCT	GGACTTCCCG	GAGCCGCTGC	GGGACCGACA	GATGGTGTCTG
421	TCCCTGGAGA	GCTGGGTGGG	CAACCGCAAC	AACACGAGGG	CCCGCAGCGT	GGTGAAGAGC
	AGGGACCTCT	CGACCCACCC	GTTCGGCTTG	TTGTGCTCCC	GGGCGTCGCA	CCACTTCTCG
481	CAGTACATCG	CCCTGGAGCT	GATGTTCTGTG	CAGAAGCTGC	CGTCCTTCGC	CGTGCTCTGGT
	GTCATGTAGC	GGGACCTCGA	CTACAAGCAC	GTCTTCGACG	GCAGGAAGCG	GCACAGACCA

Fig.9 (Cont).

```

GGCGGCCATA AGCTGGAGTT CAGGACCATC GGCGGCACCC TCAACATCAG CACCCAAGGC
CCGCCGGTAT TCGACCTCAA GTCTGTGTAG CCGCGTGGG AGTTGTAGTC GTGGGTTCCG

1141 AGCACCAACA CCAGCATCAA CCCGGTCACC CTGCCCTTCA CCAGCCGGGA CGTGATACCG
TCGTGGTTGT GGTCTGTAGTT GGCCCACTGG GACGGGAAGT GGTGGCGCT GCACATGGCG

1201 ACCGAGAGCC TGGCCGGCCT GAACCTGTTC CTGACCCAGC CCGTGAACGG CGTGCCCCCG
TGGCTCTCGG ACCGGCCGGA CTGGACAAG GACTGGGTCC GGCACCTGCC GCACGGGGCG

1261 GTGGACTTTC ACTGGAAGTT CGTGACCCAC CCGATCGCCA GCGACAACCTT CTACTACCCC
CACCTGAAAG TGACCTTCAA GCACTGSGTG GGCTAGCGGT CGCTGTTGAA GATGATGGGG

1321 GGCTACGCTG GCATCGGCAC CCAACTCCAG GACAGCGAGA ACGAGCTGCC GCCCGAGGCC
CCGATGCGAC CGTAGCCGTG GGTGAGGTC CTGTCGCTCT TGCTCGACGG CGGGCTCCGG

1381 ACCGGTCAGC CGAACTACGA GAGTACAGC CACCCCTCGA GCCACATCGG CCGTATCTCC
TGGCCAGTCG GCTTGATGCT CTCGATGTCG GTGGCGGACT CCGTGTAGCC GGACTAGAGG

1441 GCCTCCACG TGAAGGCCCT GGTGTACTCC TGGACCCACC GCAGCGCCGA CCGCACCAAC
CGAGGGTGC ACTTCCGGGA CCACATGAGG ACCTGGGTGG CGTCGGGCT GCGGTGGTTG

1501 ACCATCGAGC CGAACAGCAT CACGCAGATC CCGCTGGTGA AGGCTTCAA CCTGAGCTCC
TGGTAGCTCG GCTTGTCGTA GTGCGTCTAG GCGACCACT TCCGGAAGTT GGACTCGAGG

1561 GGTGCTGCAG TGGTSCGCG TCCAGGCTTC ACAGGCGGCG ACATCCTGCC CAGGACCAAC
CCACGACGTC ACCACGCGC AGGTCCGAAG TGTCCGCCGC GTAGGACGC GTCTGGTTG

1621 ACCGGCACCT TCGGCGACAT CCGCGTGAAC ATCAACCCCC CGTTCGCCCC GCGCTACAGG
TGGCCGTGGA AGCCGCTGTA GGCGCACTTG TAGTTGGGG GCAAGCGGGT CCGCATGTCC

1681 GTGAGGATCA GGTACGCCAG CACCACCGAC CTCAGTTCC ACACCAGCAT CAACGGCAAG
CACTCCTAGT CCATGCGGTC GTGGTGGCTG GAGGTCAAGG TGTGGTCGTA GTTGCCGTTT

```

Fig.9 i.

541 GAGGAGGTGC CCCTGCTGCC GATCTACGCC CAGGCCGCCA ACCTCCACCT CCTGCTCCTG
 CTCCTCCACG GGGACGACGG CTAGATGCGG GTCCGGCGGT TGGAGGTGGA GGACGAGGAC

 601 CGCGACGCCA GCATCTTCGG CAAGGAGTGG GGCCTGTCCT CCAGCGAGAT CAGCAGGTTT
 GCGCTGCGGT CGTAGAGGCC GTTCCTCACC CCGACAGGA GGTGCTCTA GTCGTGCAAG

 661 TACAAACAGG AGGTGGAGCG CGCCGGCGAC TACAGCGACC ATTGCGTGAA GTGGTACAGC
 ATGTTGTCCG TCCACCTCGC GCGGCCGCTG ATGTCGCTGG TAACGCACCT CACCATGTCG

 721 ACCGGCCTGA ACAACCTGAG GGGCACCAAC GCCGAGAGCT GGTCCCGCTA CAATCAGTTC
 TGGCCGGACT TGTGGACTC CCCGTGGTTG CGGCTCTCGA CCCAGGCGAT GTTAGTCAAG

 781 CGCCGGGACA TGACCCCTGAT GGTGCTGGAC CTGGTGGCCC TGTCCCGAG CTACGACACC
 GCGGCGCTGT ACTGGGACTA CCACGACCTG GACCACCGGG ACAAGGCTC GATGCTGTGG

 841 CAGATGTACC CGATCAAGAC CACCGCCCAG CTGACCCGCG AGGTGTACAC CGACGCCATT
 GTCTACATGG GCTAGTTCTG GTGGCGGGTC GACTGGGCGC TCCACATGTG GCTGCGGTAA

 901 GGCACCGTGC ACCCGCACCC GAGCTTCAG AGCACCCACT GGTACAACAA CAACGCCCCA
 CCGTGGCACG TGGGCGTGGG CTCGAAAGTC TCGTGGTGA CCATGTTGTT GTTGCGGGGT

 961 AGCTTCAGCG CCATCGAGGC CGCCGTGGTG CGCAACCCCC ACCTCCCTGGA CTTCTGGAG
 TCGAAGTCGC GGTAGTCCG CGGCACCCAC GCGTTGGGG TGGAGGACCT GAAGGACCTC

 1021 CAGGTGACCA TCTACAGCCT GCTGAGCCGG TGGAGCAACA CGCAGTACAT GAACATGTGG
 GTCCACTGGT AGATGTCGGA CGACTCGGCC ACCTCGTTGT GCGTCATGTA CTTGTACACC

Fig.9 (Cont i).

1741 GCCATCAACC AGGCAACTT CAGCGCCACC ATGAACCGCG GTGAGGACCT GGACTACAAG
 CGGTAGTTGG TCCCCTTGA GTCGCGGTGG TACTTGGCG CACTCCTGGA CCTGATGTTT

 1801 ACCTTCCGCA CCGTGGGCTT CACCAACCCG TTCAGCTTCC TGGACGTGCA GAGCACCTTC
 TGAAGGCGT GGCACCCGAA GTGTGGGGC AAGTCGAAG ACCTGCACGT CTCGTGGAAG

 1861 ACCATCGGCG CCTGGAACCT CAGCAGCGGC AACGAGGTGT ACATCGACCG CATCGAGTTC
 TGGTAGCCGC GGACCTTGAA GTCGTCGCCG TTGCTCCACA TGTAGCTGGC GTAGCTCAAG

 1921 GTGCCCGTGG AGGTGACCTA CGAGGCCGAG TAGCACTTCG AGAAGGCCCA GGAGAAGGTC
 CACGGGCACC TCCACTGGAT GCTCCGGCTC ATGCTGAAG TCTTCCGGGT CCTCTTCCAG

 1981 ACCGCCCTGT TCACCAGCAC CAACCCGCGC GGCCTGAAGA CCGACGTGAA GGACTACCAC
 TGGCGGGACA AGTGGTCGTG GTTGGGCGCG CCGGACTTCT GGCTGCACTT CCTGATGGTG

 2041 ATCGACCAGG TGAGCAACTT GGTGGAGTCC CTGAGCGACG AGTTCTACCT GGACGAGAAG
 TAGCTGGTCC ACTCGTTGAA CCACCTCAGG GACTCGCTGC TCAAGATGGA CCTGCTCTTC

 2101 CGCGAGCTGT TCGAGATCGT GAAGTACGCC AAGCAGCTGC ACATCGAGCG CAACATGTAG
 GCGCTCGACA AGCTCTAGCA CTTCATGCGG TTCGTGACG TGTAGCTCGC GTTGTACATC

 2161 GATCC
 CTAGG

Total number of bases is: 2165.

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Fig.11.

	HindIII	10	SphI	20	PstI	30	40	50	60	
1	AAGCTTGCAT	GCCTGCAGGT	CAACATGGTG	GAGCACGACA	CACCTTGTCTA	CTCCAAAAAT				
	TTCGAACGTA	CGGACGTCCA	GTTGTACCAC	CTCGTGCTGT	GTGAACAGAT	GAGGTTTTTA				
61	ATCAAAGATA	CAGTCTCAGA	AGACCAAAGG	GCAATTGAGA	CTTTTCAACA	AAGGGTAATA				
	TAGTTTCTAT	GTCAGAGTCT	TCTGGTTTCC	CGTTAACTCT	GAAAAGTTGT	TTCCCATTTAT				
121	TCCGGAAACC	TCCTCGGATT	CCATTGCCCA	GCTATCTGTC	ACTTTATTGT	GAAGATAGTG				
	AGGCCTTTGG	AGGAGCCTAA	GATAACGGGT	CGATAGACAG	TGAAATAACA	CTTCTATCAC				
181	GAAAAGGAAG	GTGGCTCCTA	CAAATGCCAT	CATTGCGATA	AAGGAAAGGC	CATCGTTGAA				
	CTTTTCCCTTC	CACCGAGGAT	GTTTACGGTA	GTAACGCTAT	TTCCCTTTCCG	GTAGCAACTT				
241	GATGCCTCTG	CCGACAGTGG	TCCCAAAGAT	GGACCCCCAC	CCACGAGGAG	CATCGTGGAA				
	CTACGGAGAC	GGCTGTCACC	AGGGTTTCTA	CCTGGGGGTG	GGTGCTCCTC	GTAGCACCTT				

DUF
ATC
EN

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Fig.11 (Cont).

301 AAAAGAAGAC GTTCCAACCA CGTCTTCAA GCAAGTGGAT TGATGTGATA ACATGGTGGA
 TTTTCTTCTG CAAGGTTGGT GCAGAAGTTT CGTTCACCTA ACTACACTAT TGTACCACCT
 361 GCACGACACA CTGTCTACT CCAAAATAT CAAAGATACA GTCTCAGAAG ACCAAAGGGC
 CGTGCTGTGT GAACAGATGA GGTTTTATA GTTCTATGT CAGAGTCTTC TGGTTTCCCG
 421 AATTGAGACT TTTCAACAAA GGGTAATATC CGGAACCTC CTCGGATTCC ATTGCCCCAGC
 TTAACCTCTGA AAAGTTGTTT CCCATTATAG GCCTTTGGAG GAGCCTAAGG TAACGGGTCG
 481 TATCTGTCAC TTTATTGTGA AGATAGTGA AAAGGAAGGT GGCTCCTACA AATGCCATCA
 ATAGACAGTG AATAACACT TCTATCACCT TTTCCCTTCCA CCGAGGATGT TTACGGTAGT
 541 TTGCGATAAA GGAAGGCCA TCGTTGAAGA TGCCCTCTGCC GACAGTGGTC CCAAAGATGG
 AACGCTATTT CTTTCCGGT AGCAACTCT ACGGAGACGG CTGTCACCAG GGTTCCTACC
 601 ACCCCCACCC ACGAGGAGCA TCGTGGAAA AAGAAGACGT TCCAACCCAG TCTTCAAAGC
 TGGGGGTGGG TGCTCCTCGT AGCACCTTTT TTCTTCTGCA AGGTTGGTGC AGAAGTTTCG
 661 AAGTGGATTG ATGTGATATC ^{EcoRV} TCCACTGACG TAAGGGATGA CGCACAATCC CACTATCCTT
 TTCACCTAAC TACACTATAG ^{Promoter} AGGTGACTGC ATTCCCTACT GCGTGTAGG GTGATAGGAA

355
PR

Fig.11 (Cont i).

721 CGCAAGACCC TTCTCTATATA TAAGGAAAGTT CATTTCAATTT GGAGAGGACC TCGAGTATTT
 GCGTTCTGGG AAGGAGATAT ATTCTTCAA GTAAAGTAAA CCTCTCCTGG AGCTCATAAA
 TATA BOX
 781 TTACAACAAT TACCAACAAC AACAAACAAC AAACAACATT ACAATTACTA TTTACAATTA
 AATGTTGTTA ATGGTTGTTG TTGTTTGTG TTTGTTGTAA TGTTAATGAT AAATGTTAAT
 841 CACCATGGAT CCCCAGGTAC CGAGCTCGAA TTTCCCCGAT CGTCAACA TTTGGCAATA
 GTGGTACCTA GGGGCCCATG GCTCGAGCTT AAAGGGGCTA GCAAGTTTGT AAACCGTTAT
 NOS
 901 AAGTTTCTTA AGATTGAATC CTGTTGCCGG TCTTGCGATG ATTATCATAT AATTTCTGTT
 TTCAAGAAT TCTAACTTAG GACAACGGCC AGAACGCTAC TAATAGTATA TTAAAGACAA
 961 GAATTACGTT AAGCATGTAA TAATTAACAT GTAATGCGATG ACGTTATTTA TGAGATGGGT
 CTTAATGCAA TTCGTACATT ATTAATTGTA CATTACGTAC TGCAATAAAT ACTTACCCA
 1021 TTTTATGATT AGAGTCCCGC AATTATACAT TTAATACGCG ATAGAAAACA AAATATAGCG
 AAAATACTAA TCTCAGGGCG TTAATATGTA AATTATGCGC TATCTTTTGT TTTATATCGC
 1081 CGCAAACTAG GATAAATTAT CGCGCGCGGT GTCATCTATG TTACTAGATC GCGAATTC
 GCGTTTGATC CTATTTAATA GCGCGCGCCA CAGTAGATAC AATGATCTAG CCCTTAAG
 EcoRI

Total number of bases is: 1138.

DNA sequence composition: 370 A; 253 C; 234 G; 281 T; 0 OTHER;

Sequence name: PMJB1

2 2 / 2 2

Fig.12.

